

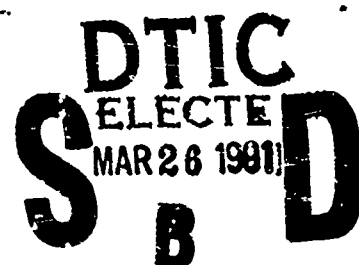
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CHARACTERIZATION OF AN OPIOID-LIKE
HIBERNATION INDUCTION TRIGGER

ANNUAL/FINAL REPORT

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<p>A hibernation induction trigger (HIT) molecule derived from the plasma of deeply hibernating woodchucks exerts profound effects mimicking a hibernation-like state when infused I.C.V. or I.V. in primates. The profound opiate-like behavioral and physiological depression occurring shortly after the infusion of the HIT-containing albumin fraction include hypothermia, bradycardia, long-term hypophagia and markedly depressed renal function. All of the aforementioned effects are reversed or retarded by the infusion of the opiate antagonists, naloxone and naltrexone. Such evidence enforces our hypothesis that the HIT molecule is not specific for hibernators, but that it may initiate its action through specific opioid receptor(s). We have shown that only DADLE, a delta class of opioid, could induce hibernation in summer-active ground squirrels (the traditional bioassay) in a fashion similar to those injected with HIT. Such evidence indicates that this delta opioid mimics the action of the HIT molecule and may be intimately involved in natural winter and summer-induced hibernation. Recently, we have developed a rapid</p>					
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in vitro bioassay utilizing three criteria (DNA synthesis inhibition, protein synthesis inhibition and intracellular calcium release) to monitor various purified fractions for winter HIT-inhibitory or summer mitogenic activity and to determine the effects of these molecules on cellular metabolism. Moreover, we now have evidence that the clinical potential for these molecules especially in combat or surgical trauma related situations may be vast. We have clearly shown that both HIT and DADLE can prolong organ survival time by over 3-fold in our newly developed multiorgan autoperfusion system. The mechanism(s) by which HIT or DADLE increased tissue survival time in these preliminary studies revealed three possible beneficial effects: 1) they may markedly reduce tissue metabolism, 2) they may reduce or eliminate platelet and/or leucocyte aggregation (associated with microthromboemboli in vital organs such as liver, lung, kidney, and heart), and 3) they may improve microcirculation via vasodilation. If these effects can be duplicated and confirmed utilizing a fully characterized HIT molecule, it is possible that in the future HIT and DADLE and perhaps other delta opioids could have major clinical significance in the treatment of trauma, microvascular disease, multiple organ system failure, tissue preservation, and cryogenic surgical procedures.

FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council (DHHS, PHS, NIH Publication No. 86-23, Revised 1985).

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INTRODUCTION

Dawe and Spurrier (Dawe et al.¹) were the first investigators to present evidence for the presence of a hibernation "trigger" in the plasma of hibernating thirteen-lined ground squirrels (Citellus tridecemlineatus) which could induce a similar state when injected into either summer-active ground squirrels or woodchucks (Marmota monax) (Dawe et al.²). However the delineation of the chemical identity of this hibernation "trigger" has been relatively slow until quite recently. This may primarily be attributed to the necessity of utilizing a bioassay requiring induction of hibernation in summer-active ground squirrels or woodchucks (a very restrictive seasonal time frame for testing). In spite of this major drawback, our work over the past years, utilizing a variety of protein resolving techniques including isoelectric focusing (Haglund³), preparative isotachopheresis (Hjalmarsson⁴) and affinity chromatography (Travis et al.⁵) has given the first real clues to the chemical identity of the "trigger" molecule which has been termed the hibernation induction trigger or HIT-molecule, an acronym that is now established in the literature. Our experiments have demonstrated that this molecule is closely bound to or associated with albumin and that its physiological role in hibernators may be dependent upon changing albumin concentrations (Oeltgen et al.⁶). To date, our studies indicate that the HIT molecule is a small, thermolabile, protease-sensitive, nuclease-insensitive protein possibly in excess of 5,000 M.W. (Oeltgen et al.⁷). Furthermore, the studies of Spurrier et al.⁸, Oeltgen et al.⁹ and Rotermund et al.¹⁰ have demonstrated biochemical and physiological effects of the HIT molecule on the circulation, blood, and membrane components of hibernators which favor survival in the cold. Among the changes noted were increased resistance of erythrocytes in osmotic fragility tests (Spurrier et al.⁸), the inability to agglutinate (human or ground squirrel) cells in ground squirrel serum at low temperature (Spurrier et al.⁸) and the higher degree of unsaturation of fatty acids in erythrocyte membranes¹⁰, (resulting in enhanced membrane fluidity) which make them more pliable and less susceptible to rouleau formation in constricted capillary beds of these hibernating animals whose core temperature may be close to freezing.

Although the HIT molecule has been shown to be highly effective in specially adapted hibernating species, our primate studies indicated that intracerebroventricular (ICV) infusion in conscious chaired primates of the isolated HIT-containing albumin fraction from hibernating woodchucks can produce opiate-like behavioral modifications¹¹ and initiate profound behavioral and physiological depression resembling a hibernating-like state including hypothermia, bradycardia and long-term hypophagia (Oeltgen et al.¹², Myers et al.¹³). Intravenous infusions of an albumin fraction derived from winter-hibernating but not summer-active woodchucks initiates quite comparable responses to the aforementioned ICV infusions and dramatically alters primate renal function as evidenced by marked decreases in creatinine clearance and urine flow (Oeltgen et al.¹⁴).

Since most of the aforementioned behavioral and physiological depressions noted in primates can be blocked or retarded by the I.V. or I.C.V. infusion of opiate antagonists, naloxone and naltrexone, we now have reason to suspect that the HIT molecule is either opiate in nature or a neuropeptide hormone which initiates its action through an opioid

receptor(s) (Oeltgen *et al.*¹²). We have also shown that the opioid antagonist naloxone (Bruce *et al.*¹⁵), the kappa agonist U69593 (Oeltgen *et al.*¹⁶) and the mu agonist, morphine and morphiceptin, as well as the naturally occurring kappa brain opioid agonist, dynorphin, can all block hibernation induced by HIT when infused via mini osmotic pumps which have been implanted subscapulary in summer-active ground squirrels (Su *et al.*¹⁷, Oeltgen *et al.*¹⁸). Only the delta opioid D-Ala²-D-Leu⁵-Enkephalin (DADLE) induced hibernation in summer-active ground squirrels in a fashion similar to that observed in animals injected with HIT and saline in the infusion pumps (Su *et al.*¹⁷, Oeltgen *et al.*¹⁸). We have recently completed development of a rapid *in vitro* assay system which replaces the seasonally restrictive ground squirrel bioassay with the intention of utilizing it for both the purification and characterization of HIT-like molecules present in hibernating woodchuck plasma. This system measures inhibition of DNA and protein synthesis (metabolic inhibition at the cellular level) and unusual changes in calcium levels in cultured cells exposed to these molecules. Three cell lines are used for this *in vitro* bioassay system: 1) TRMP-a dog kidney epithelial cell line (Turker *et al.*¹⁹) 2) CREF-a rat embryo fibroblast cell line (Pozzati *et al.*²⁰), and 3) SB3-a human kidney tumor (Wilm's) cell line.

Moreover, in a continuing effort to fully characterize the HIT molecule and its opioid related derivatives, we have carried out a unique series of experiments utilizing a newly developed autoperfusion multiorgan preservation system (Chien *et al.*²¹, Chien *et al.*²²). We have the first experimental evidence which illustrates vast clinical potential for the HIT molecule and the delta opioid, DADLE, which mimics the activity of HIT in hibernators, in dramatically extending effective organ preservation time (Chien *et al.*²³, Chien *et al.*²⁴, Nilekani *et al.*²⁵). The heart-lung autoperfusion system was first used for organ preservation study by Robicsek *et al.*^{26,27} and we have refined and expanded the preservation of organs to include the liver, pancreas, duodenum and both kidneys. While still being perfused by the heart and oxygenated by the lungs, the heart, lungs, liver, pancreas, duodenum and both kidneys are removed en bloc and placed in a bath solution. No anticoagulation is needed and no inotropic drugs are necessary. It is a self-contained unit with independent physiological functions without any foreign materials. The heart pumps the blood; the lungs oxygenate the blood; the kidney adjusts water and electrolyte balance and removes metabolic waste products, while the liver maintains its biological function including energy metabolism and production of coagulation factors. All the major organs are preserved with this relatively simple autoperfusion preparation. In our multiorgan preservation study, it was found that the inferior vena cava contained contrast particles, as seen by ultrasound imaging, which moved with the blood flow and appeared to increase in size and number during the course of preservation. Ultrasonic detection of large numbers of particles in perfusate during cardiac surgery, as a result of cardiopulmonary bypass procedure, has previously been associated with multiorgan failure and a poor prognosis (Mahony *et al.*²⁸, Mahony *et al.*²⁹). Serial lung sections showed clumps of white cells in the vascular space. These two findings suggest the possibility that one reason for progressive organ dysfunction during the autoperfusion studies might occur from an embolization by platelet and neutrophil aggregates in the heart, lungs, liver and kidney. Our preliminary findings with HIT and DADLE, although not in a large scale, could provide a clue for the possible mechanism of multiple organ failure. In spite of many studies, the etiology, mechanism and treatment of multiple organ failure (MOF) or multiple system organ failure (MSOF) are still not clear. The mortality rate in patients with MOF can be as high as 90

percent. MOF occurs more often after major trauma (as seen in battle field) or major surgery. If the aggregated platelet and/or leukocyte is related to the development of MOF, and HIT or DADLE can reverse this aggregation, it would be possible to use HIT or DADLE for the treatment of MOF and save many seemingly severely damaged organs and body parts by blocking or reversing the formation of these aggregates throughout the circulatory system.

MATERIALS AND METHODS

A. SAMPLE PROCUREMENT

1. **HIT-Containing Plasma:** Plasma to be assayed for HIT activity was obtained from woodchucks weighing 3.0 to 5.0 kg maintained in an 8' x 12' hibernaculum at 4-6° C and from hibernating and summer-active black bears maintained in enclosures with dens by Dr. Ralph Nelson at the University of Illinois, Urbana, IL. Blood from woodchucks was drawn aseptically by heart ventricular puncture while these animals are in deep hibernation as evidenced by having a core temperature of approximately 5° C and heart rates of one to two beats per minute. Normally it takes these hibernators one or two hours to arouse (core temperature reaching 37° C and heart rate returning to normal) after this painless blood drawing procedure. Blood from black bears is obtained from the femoral artery after both active and hibernating animals are anesthetized with ketamine and Rompum. The bears' core temperature drops only 2 or 3° C even when hibernating and arousal can occur in minutes with anesthesia.

2. **DADLE** - (Tyr-D-Ala-Gly-Phe-D-Leu) was purchased from Peninsula Laboratories, Belmont, CA.

DADLE is a biologically stable structural analog of "ENKEPHALIN". ENKEPHALIN means: brain (ENKE) morphine (PHALIN), meaning a morphine-like substance in the brain which is made from naturally existing materials in the brain---amino acids. There are two kinds of naturally-occurring enkephalins, methionine-enkephalin and leucine-enkephalin. The enkephalins, however, are biodegradable. Specific enzymes like enkephalinase and angiotensin converting enzyme can degrade enkephalins. Therefore, enkephalin analogs resist to enzymatic degradation were synthesized. Many enkephalin analogs resistant to enzymatic degradation were synthesized. Many enzymatically stable enkephalin analogs are available nowadays. To date DADLE [(D-Ala²,D-Leu⁵)enkephalin] remains the most known stable enkephalin analog.

For the multiorgan autoperfusion studies, DADLE was infused into intact dogs or the multiorgan bloc at a concentration of 1 mg/kg which is a typical effective dosage for the opioids.

B. BIOASSAYS EMPLOYED

1. **In-Vivo Bioassay for HIT:** Induction of summer hibernation during the months of June, July, August and the middle of September was the assay method that was used to test whole

plasma and fractionated plasma samples for HIT activity. A group of nontransfused animals was maintained under similar conditions as the experimentals. Control groups were injected with 0.9% saline vehicle or plasma fractions from summer-active woodchuck plasma which have undergone similar analytical separations as those derived from winter hibernating woodchuck plasma. Six-month old, summer-active ground squirrels, weighing 160-260 g and maintained in the warm room at 23°C are the test animals (5-8 animals per group) for woodchuck and bear plasma fractions to be assayed for HIT activity. These fractions maintained at 4°C are injected into the saphenous vein of ground squirrels at a concentration of 5 mg protein/500 ul of 0.9% saline and the animals are immediately placed in the 5°C hibernaculum.

2. In-Vitro Bioassay: A dog kidney cell line (TRMP) and a rat embryo fibroblast line (CREF) were used in the study. Cultures of each cell line were grown and maintained at 37° C in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 5% fetal bovine serum (FBS). TRMP cells (7×10^4 cells/well) and CREF cells (5×10^4 cells/well) were plated in 24-well microtitre culture plates. Twenty four hours later each well was washed once with 2 ml of serum free DMEM and cells maintained in serum free DMEM (2 ml) for 24-30 hours. All experiments were run in duplicates.

Incorporations of ^3H -Thymidine in serum or plasma stimulated cells. Serum starved cells were stimulated with 5% FBS and/or different concentrations of summer and winter woodchuck plasma. For these experiments the incorporation of ^3H -thymidine in the cells was monitored in two hour pulses from 8-22 hours after stimulation. At the end of the two hour pulse, the medium was gently removed from the wells and the cell sheet was washed twice with cold PBS. Cells were solubilized with 500 ul of 0.2 N NaOH followed by the addition of 5 ul of 5 mg/ml salmon sperm DNA as a carrier DNA. The microtitre plates were left at 37° C for 45-60 mins before transferring the contents of each well to 1.5 ml microcentrifuge tubes. Macromolecules were precipitated with ice-cold 40% TCA containing 60 mM Na pyrophosphate. After centrifugation the cell pellet was washed once with 10% TCA and dissolved in 200 ul of 0.2N NaOH. The relative level of ^3H -thymidine incorporation was determined by liquid scintillation counting.

Intracellular calcium changes were measured by Dr. Jesse Siskin's laboratory by preloading cells with aequorin via the scrape loading technique (Fechheimer *et al.*³⁰) and measuring aequorin-luminescence which is dependent upon the relative amounts of free calcium in the cells (McNeil *et al.*³¹).

C. ANALYTICAL TECHNIQUES EMPLOYED IN ISOLATING AND CHARACTERIZING THE HIT MOLECULE(S)

1. Protein Determinations: Protein content of all the resolved plasma fractions will be determined using the method of Lowry *et al.*³² as modified by Oyama and Eagle³³.

2. Affinity Chromatography: The affinity chromatographic technique, utilizing Affi-Gel Blue (Bio-Rad Industries, Richmond, CA) as the chromatography matrix was utilized to rapidly

obtain sufficient quantities of nearly homogeneous HIT-containing albumin fractions which were then subjected to further isolation procedures. This technique has proven highly effective in selectively absorbing albumin from plasma of hibernating or summer-active woodchucks³⁵ for experiments in which induction of hibernation in summer-active ground squirrels was used as the bioassay for HIT activity. In this technique, carried out at 4°C, the affinity column is loaded with up to 10 ml of lyophilized plasma from hibernating or summer-active woodchucks dissolved in 0.02 M Na-phosphate buffer pH 6.8 and the non-albumin fraction (g-globulins) was eluted with this buffer at pH 5.7. A high concentration of salt 1.4 M NaCl, added to the pH 5.7 buffer is then utilized to desorb the HIT-containing albumin fraction from the gel matrix. The column was then regenerated with two bed volumes of 6 M guanidine HCL. The homogeneity of 400 ug aliquots of all these fractions was determined by analytical polyacrylamide gel electrophoresis procedure utilizing the standard methodology of Davis³⁴. Gels were stained with Coomassie Blue as we have previously described³⁵. Prior to IV or ICV infusions these fractions are rendered sterile by passing them through a 0.21 µm Amicon Sterilet (Amicon, Danvers, MA).

D. DEFINING THE OPIOID NATURE OF THE HIT MOLECULE

To examine the possible involvement of multiple opioid receptors in animal hibernation, we infused opioids selective for mu, kappa, and delta opioid receptors into summer-active ground squirrels. These studies were conducted in two phases and involved the use of 92 ground squirrels which were caught wild in the summer-active non-breeding condition weighing 160-220 g. Both females and males were used. Osmotic pumps (Alzet Model 2ML4, Alza Corp., Palo Alto, CA) containing either physiological saline or the mu, kappa, and delta opioid agonists were inserted subscapularly and held in place by a wound clip.

In the first study, animals were divided into three groups. Each control group A animal (n=5) was injected in the saphenous vein with 10mg of HIT-containing albumin fraction and the osmotic pumps were filled with 2,158 ul (SD ± 70 ul) of saline and were inserted subscapularly. Each group B animal (n=8) was injected with 10 mg of HIT on 0.5 ml of saline and the osmotic pump was filled with 3.3 mg of U-69593 dissolved in 2,158 ul of saline, these were also inserted subscapularly. Based on a mean pumping rate of 2.58 ul/hr or 62 ul/day, each ground squirrel received a dose of approximately 0.475 mg U-69593/kg/day. Each group C animal (n=7) was injected with 0.5 ml saline and the osmotic pump was filled with the aforementioned concentrations of U-69593 and inserted subscapularly.

The animals were maintained in a hibernaculum at 4-6° C and observed at two day intervals for hibernation. Hibernating animals exhibits piloerection of fur and remained in a balled-up position in a nest surrounded by bedding. Moreover, respiration in hibernating animals slowed from over 100 to 8-10 respirations per minute and the animals did not respond to external stimuli (light and sound).

In the second study, 72 ground squirrels were utilized and divided randomly in equal numbers into nine groups. One group served as an overall control group which received only saline in the osmotic minipumps. Osmotic minipumps containing the following opioids were

inserted subscapularly into the rest of the animals, 16 animals per drug: morphine sulfate (Merck, Piscataway, NJ), morphiceptin, dynorphin A (1-13) and DADLE (Peninsula Laboratories, Belmont, CA). Eight animals per drug group then received an intrasaphenous injection of either saline or HIT (10 mg lyophilized preparation or 5 mg protein in 500 ul saline). Based on a mean pumping rate of 2.58 ul/hr or 62 ul/day, each ground squirrel received a dose of approximately: 1.50 mg/kg/day morphine, 0.82 mg/kg/day morphiceptin, 0.82 mg/kg/day dynorphin A (1-13) and 1.50 mg/kg/day DADLE. The doses were based partially on calculations from relative affinities of those drugs at different opioid receptors in several different studies¹⁵. An arbitrarily chosen reference was the kappa opioid U69593 at 0.475 mg/kg/day. This opioid has been shown to exert a pharmacological effect on HIT-induced hibernation¹⁵. These animals were observed for hibernation as previously described for the first group.

E. MULTIORCAN AUTOPERFUSION SYSTEM

1. **Animals studied.** Twenty-one adult mongrel dogs weighing 17 to 30 kg of either sex were used in these studies. The HIT study group consisted of 7 dogs in which hibernation induction trigger(s) (HIT) containing plasma was given before and after the operation. The control group consisted of 8 dogs in which no HIT or DADLE were given.

The DADLE study group consisted of 6 dogs in which DADLE was infused before and after the operation. In order to obtain normal organ wet/dry weight ratio, tissue samples from the heart, lungs, liver, pancreas, duodenum and kidney were taken from ten normal dogs and used for comparison.

2. **Pretreatment.** All the dogs were given neomycin 2 gm, orally, once a day for three days before the operation in order to sterilize the digestive system. The dogs were fasted 10 hours before the operation. In the HIT study group, 10 ml of plasma containing hibernation induction trigger (HIT), which was obtained by intraventricular puncture from deeply hibernating woodchucks was injected intravenously 2 hours prior to the operation. The control group received 10 ml of sterile physiological saline. In the DADLE study group, the animals were infused with DADLE at a concentration of 1 mg/kg 2 hours prior to the operation in 5 ml physiological saline. No DADLE was given to the control group which was infused with physiological saline

3. **Surgical Technique.** Briefly, the dog was anesthetized with sodium pentobarbital 20 mg/kg, intubated and artificially ventilated. In the supine position, the abdomen was opened and the liver, pancreas, duodenum, both kidneys, abdominal aorta, and inferior vena cava were dissected free. The spleen was removed and one catheter was placed in the splenic artery for arterial blood pressure measurement. Another catheter was placed in the portal vein, via a splenic vein insertion, for blood and fluid transfusion. The common bile duct and the duodenum were cannulated for fluid collections. Two catheters were placed in the ureters for urine collection. The chest was then opened through a medium sternotomy. The diaphragm was incised around the liver. The heart, lungs, aorta, and superior and inferior venae cavae (SVC and IVC) were dissected free.

A catheter was placed in the left ventricle (LV) through the carotid artery for LV pressure and dp/dt measurements. Another catheter was placed in the IVC for central venous pressure (CVP) measurement and blood sampling.

The whole system was then removed from the body and placed in a water bath containing lactated Ringer's solution, heparin 10 mg/L, neomycin 0.1 g/L and penicillin 100,000 units/L.

4. Interventions. The temperature was maintained around 32°C by heating the water bath with a constant temperature circulator. Artificial respirator at a tidal volume of 500-700 ml, a rate of 10-20 rpm and PEEP of 2-6 cm H₂O. A gas mixture of 50% O₂ + 3% CO₂ + 47% N₂ was utilized. The following solutions were given at 10 to 20 ml/hour through the portal vein: dextrose (5%), calcium chloride (1 g/L), insulins (50 units/L), mannitol (12.5 g/L), methylprednisolone (500 mg/L), penicillin (1,000,000 units/L), and Flagyl (500 mg/L).

Another 5% dextrose solution containing potassium chloride, 0.5 g, was infused slowly through the portal vein to maintain serum potassium at a normal level.

A fat emulsion (Soyacal) 2 ml, and methylprednisolone 30mg, were given through the portal vein every 2 hours. Blood transfusions were given to maintain aortic systolic pressure between 75 and 100 mmHg, and CVP between 0 and 10 mmHg. Plasma was given instead of whole blood if the hematocrit was higher than 45%.

5. Application of HIT. Four ml HIT-containing plasma, which was obtained from deeply hibernating woodchucks, was given through the portal vein every 4 hours during the preservation period in the study group. Four ml of physiological saline was infused in the control group during the preservation period.

6. Application of DADLE. DADLE at concentration of 1 mg/kg in 1 ml of physiological saline was infused through the portal vein every 24 hours during the preservation period in the study group.

7. Monitoring. Aortic pressure, left ventricular pressure, dp/dt, central venous pressure, portal venous pressure and aortic blood flow were monitored and recorded on a Sensor/Medics R612 Dynograph Recorder throughout the preservation period. Temperature, urine output, bile production and duodenal and pancreatic secretions were collected and recorded every hour. Visual changes including color, size and bleeding for each organ, respiratory pressure, tidal volume and PEEP for the lungs were recorded every hour. Arterial blood gas and hematocrit measurements were taken before the operation and every 4 hours during the preservation period and used for blood chemistries, hematology, lactic acid and enzyme measurements for heart, liver, pancreas and kidney functions. Tissue samples were taken from the lungs every 8 hours for tissue wet/dry weight ratio and electron microscope studies. At the termination of the study, specimens were taken from each organ for wet/dry ratios and pathologic examinations.

8. Determination of Tissue Wet/Dry Weight Ratio. Tissue samples used for wet/dry ratio measurement were blotted to remove excess fluid and wet weight was measured. The dry weight was determined after the samples had been in an oven at 85° C for 72 hours.

9. Statistical Analysis. All the laboratory tests obtained before the operation (blood gases, hematocrit, blood chemistries, hematology, lactic acid and enzymes for heart, liver, pancreas and kidney functions) were used as normal controls which were compared to the parameters obtained during the preservation period. Heart rate, blood pressures, left ventricular dp/dt, blood gases, urine output, comparisons were made between those obtained immediately after harvesting and those obtained during the preservation period. Tissue wet/dry weight ratios for all the organs were compared with those obtained from the normal control dogs.

Paired and unpaired student's t-tests were used to compare the parameters measured during the preservation period with those obtained preoperatively or immediately postoperatively. The level of significance was 0.05.

RESULTS

A. DEFINING THE OPIOID NATURE OF THE HIT MOLECULE(S)

The inhibitory action on summer-induced hibernation (the traditional bioassay) by the opioid antagonist, naloxone is shown in Fig 1 while that of the kappa agonist, U-69593 and dynorphin and the mu agonists morphine and the morphiceptin is depicted in Fig. 2-4. The hibernation induction capacity of the delta opioid, DADLE, is depicted in Figs. 5 and 6. Only the delta opioid, DADLE, induced hibernation in summer-active ground squirrels in a fashion similar to those injected with HIT.

B. DEVELOPMENT OF A RAPID IN VITRO BIOASSAY TO FACILITATE PURIFICATION OF THE HIT MOLECULE AND RELATED FACTORS

To monitor inhibition of DNA synthesis, two separate assays have been used. In the first, the cells are serum starved for 24 hours and then restimulated with 5% fetal bovine serum (FBS) in the presence of various concentrations of plasma obtained from winter-hibernating or summer-active woodchucks. Fig 7 depicts results with the TRMP cells and Fig 8 with the CREF cells demonstrating that complete to partial inhibition of the FBS stimulated DNA synthesis was observed with the winter plasma (WWP). For the CREF cells, even at a concentration as low as 0.5% winter-hibernating plasma, significant inhibition of FBS DNA synthesis was observed. For both cell lines, equivalent concentrations of the summer-active plasma (WSP) enhanced the FBS stimulation of DNA synthesis. For the second assay, continuously growing cells were exposed to various concentrations of winter plasma and DNA synthesis measured every hour for six hours. Fig 9 demonstrates with the CREF cells that DNA synthesis slowed and eventually halted in these cells in a concentration dependent manner. Although not shown in this figure, the summer-active plasma did not inhibit DNA synthesis. To monitor inhibition of protein synthesis, continuously growing cells were also used. The winter plasma was found to inhibit protein synthesis but equivalent amounts of summer plasma did not. Fig. 10 demonstrates this for the TRMP cells. The above results with serum starved cells stimulated with FBS and summer plasma suggested that the summer plasma may also play a role in the stimulation of DNA synthesis (see Figs. 7 and 8). To test this hypothesis, we stimulated serum starved TRMP cells with concentration of summer plasma ranging from 0.5%-5.0% in the absence of FBS and found significant stimulation (not shown). This demonstrated that the woodchuck summer-active plasma contained a potent mitogenic activity. The detection of such activity in the summer plasma was quite surprising since plasma from most species, relative to serum, is nitrogen poor (Balk *et al.*³⁶). Identical experiments with winter-hibernating plasma demonstrated that at concentrations as low as 2%, DNA synthesis did not occur in the TRMP cells. However, when the plasma concentration was dropped to 0.5%, the winter plasma displayed significant mitogenic activity. Fig 11 demonstrates that both summer-active and winter-hibernating plasma could stimulate

detectable amounts of DNA synthesis in the TRMP cells at concentrations as low as 0.01% whereas FBS could not stimulate DNA synthesis at concentrations below 0.25%.

When serum starved cells are restimulated with FBS the first measurable response is a transient intracellular release of stored calcium (McNeil *et al.*³¹). This response occurs within seconds, followed by a biphasic pump down of the free intracellular calcium. (Fig. 12A) demonstrates the calcium response of serum starved TRMP cells to 10% FBS, (Fig.12B) to 10% winter-hibernating plasma, and (Fig12C) to 10% summer-active plasma. Several significant observations were noted. The winter plasma stimulated an initial intracellular calcium release similar to that observed for the FBS, but this was followed by a second, prolonged peak representing accumulation of intracellular calcium. We have now tested a number of winter-hibernating plasmas and this second calcium peak was always observed. The summer plasma stimulated a greater intracellular calcium release than either FBS or winter plasma and was also followed by a second calcium peak. However, this second peak was significantly lower than that observed for the winter plasma.

With the establishment of this *in vitro* system, we have begun work to determine if this system will function as a bioassay for the purification of both the inhibitory (HIT-active) and mitogenic activities present in the woodchuck plasmas. Both summer-active and winter-hibernating plasmas were passed over an Affi-Gel Blue affinity column and three fractions collected: the pass through, proteins removed from the column with 0.2M sodium phosphate, and proteins eluted with 1.4 M NaCl. The last fraction was dialyzed after collection. Table 1 demonstrates that the bulk of the inhibitory activity from the winter-hibernating plasma eluted with high salt. This fraction is enriched from albumin (Oeltgen *et al.*⁸). Table 2 demonstrates that the bulk of the mitogenic activity from both the summer-active and winter-hibernating plasma also eluted in this peak, although significant amounts were also found in the other fractions when the TRMP cells were used as a target. We have not yet tested these fractions for inhibition of protein synthesis or their effect on calcium release.

C. POTENTIAL CLINICAL APPLICATION OF HIT AND DADLE IN THE MULTIORGAN AUTOPERFUSION SYSTEM

The multiorgan autoperfusion system utilized in our studies is depicted in Fig 13. When HIT-containing plasma from deeply hibernating woodchucks was infused into the multiorgan autoperfusion system, the survival time was extended more than three-fold with an average survival time of 43.4 hours (ranging from 33 to 56 hours) under non-sterile conditions (Figures 14 A & B).

Parameters Monitored in the Multiorgan Autoperfusion System

Cardiac Function. In the study group, aortic systolic pressures (AOSP) ranged from 64±2 mmHg to 92±58 mmHg and were easily adjusted by blood or plasma infusion. No inotropic drugs were necessary.

Aortic diastolic pressure ranged from 33 ± 7 to 58 ± 12 mmHg. Aortic pulse pressure ranged from 27 ± 3 to 36 ± 2 mmHg and did not fluctuate appreciably during the preservation period. (Immediately after the operation it was 30 ± 4 mmHg and at 40 hours, it was 28 ± 11 mmHg). CVP ranged from 3.0 ± 1.2 to 8.7 ± 3.2 mmHg. Heart rate ranged from 80 ± 7 to 103 ± 10 beats per minute (Fig. 15). Left ventricular maximum dp/dt ranged from 750 ± 353 to 1775 ± 225 mmHg/sec. Calculated maximum dp/dt/p ranged from 11.05 ± 4.60 to 24.14 ± 2.00 (sec^{-1}) (Fig. 16). In the control group, heart function was well maintained during the short preservation period although the survival time was much shorter than the study group.

Heart functions deteriorated 1 to 2 hours before overall failure occurred in the system. If severe infection developed, the heart deteriorated much faster. In the study group, the heart was more sensitive to hyperkalemia. In 3 experiments, sudden heart arrest happened when blood potassium level raised above 6 mmol/L which caused premature death of the whole system even though all other organs were still in good condition.

Lung Function. When a gas mixture of 50% O_2 + 3% CO_2 + 47% N_2 was used during the preservation period, arterial oxygen tension (PaO_2) ranged from 180 ± 35 to 285 ± 6 mmHg. Carbon dioxide tension (PaCO_2) ranged from 21 ± 2 to 32 ± 4 mmHg. Arterial pH values ranged from 7.28 ± 0.07 to 7.46 ± 0.05 (Fig. 17). Lung color changes started earlier than functional changes. However, the lungs maintained good function for more than 40 hours. Lung wet/dry weight ratio was 5.20 at the beginning. It changed to 5.46 at 32 hours and increased to 6.13 at 40 hours.

Liver Function. Total bile output during the preservation period ranged from 150 ml to 350 ml with an average of 4.7-5.9 ml/hour. A distinctive difference was found between the study group and control group. The liver began to deteriorate as early as the start of the abdominal operation in the control group. This included swelling, patchy darkening, stiffness to the touch and sweating from the surface. Liver congestion was so severe that sometimes an inotropic drug was necessary in order to maintain a satisfactory arterial blood pressure. The change became worse as the preservation continued. After 12 hours, most of the livers were enlarged, pale and stiff. In the study group, however, the livers had minimal change. In some experiments, they showed signs of congestion, including patchy darkening during the operation. It gradually returned to normal during the preservation period after infusing HIT. Laboratory tests for liver function showed increases in SGOT, SGPT, LDH and ALP immediately after the operation. They decreased during the preservation period and tended to increase again after 36 hours.

The increase was earlier and more severe in the control group (Fig. 18, 19).

Pancreatic and Duodenal Function. The pancreas and duodenum had minimal changes during the preservation period. Secretions from these organs ranged from 3-9 ml/hour (total 130 to 450 ml). Blood amylase levels did not change during the preservation period (Fig 20).

Renal Function. Total urine output ranged from 800 to 2400 ml during the preservation period with hourly urine output averaging from 15 to 70 ml in the study group. In the control group, total urine output ranged from 13 to 650 ml with an average of 0.6 to 54 ml per hour.

Kidney function was well preserved during the preservation period in the study group. Blood urea nitrogen (BUN) was reduced to about 40% of the starting levels during the preservation period ($p < 0.0005$ at 24 hours). Blood creatinine levels decreased by 70% at 8 hours ($p < 0.025$) and maintained a significantly lower level throughout the experiments ($p < 0.025$ at 24 hours) (Fig 21). In the control group, premature renal failure occurred in two experiments. Both BUN and creatinine also decreased while the kidney was working.

Hematology Study. Stable RBC concentrations were maintained during preservation by blood or plasma transfusions. Heparin was not used therefore bleeding was not a big concern even though the dissection was extensive. RBC concentrations tended to increase due to the exudation of lymph. Plasma infusion was always necessary in all experiments to keep hematocrit at normal levels. WBC counts had a continuous decrease during the preservation period in both groups ($p < 0.0005$ at 12 and 24 hours). Blood platelet levels had slight decreases during the preservation period but was not statistically significant (Fig. 22). Free plasma hemoglobin had only a two-fold increase at 24 hours in the study group. The increase was severe in the control group (Fig. 23).

Blood Chemistry. Serum potassium, calcium and glucose were replaced as needed to maintain normal levels. Serum potassium tended to decrease due to high volume urine output. However, a relatively lower serum potassium did not cause much trouble. Sodium and chloride levels remained in the normal range during the preservation period in both groups (Fig. 24).

Pathology Study. Lung tissue samples were studied by electron microscope and good tissue preservation up to 40 hours was revealed. There was moderate interstitial widening caused by edema at 40 hours. Good tissue preservation was also noted from the heart, liver, pancreas, duodenum and kidney at 36-44 hours.

Comparable multiorgan survival times averaging 46.7 hours (ranging from 41 to 60 hours) were achieved when the autoperfusion system was infused with the delta opioid, DADLE, which mimics the natural biological activity of the winter hibernating plasma (Fig 25A & B). Similar to the HIT studies cardiac lung, renal, pancreatic, hepatic, hematological and blood chemistry function were well maintained compared to controls. In the DADLE-treated group, aortic systolic pressures ranged from 62 ± 6 mmHg to 79 ± 53 mmHg and was easily adjusted by blood or plasma infusion. No inotropic drugs were necessary. Aortic diastolic pressure ranged from 33 ± 44 to 49 ± 7 mmHg. Aortic pulse pressure ranged from 23 ± 4 to 30 ± 6 mmHg and did not fluctuate appreciably during the preservation period. CVP ranged from 4.9 ± 0.6 to 9.2 ± 2.9 mmHg. Heart rate ranged from 86 ± 9 to 100 ± 12 beats per minute. Left ventricular maximum dp/dt ranged from 1250 ± 353 to 1920 ± 163 mmHg/sec. Calculated maximum dp/dt/p ranged from 17.2 ± 2.3 to 25.4 ± 4.5 (sec⁻¹).

Arterial oxygen tension (PaO_2) ranged from 264 ± 32 to 348 ± 9 mmHg. Carbon dioxide tension (PaCO_2) ranged from 13.6 ± 2.4 to 23.8 ± 6.1 mmHg. Arterial pH values ranged from 7.29 ± 0.07 to 7.48 ± 0.08 . Lung wet/dry weight ratios were 5.40 at the beginning. It changed to

5.81 at 24 hours and increased to 6.35 at 40 hours. Total bile output during the preservation period ranged from 122 ml to 300 ml with an average of 2.7-6.1 ml/hour. In the control group, total urine output ranged from 13 to 800 ml (0.6-59 ml/hour). In the study group, blood urea nitrogen reduced from 13.86 to 6.17 mg/dL at 44 hours. Blood creatinine levels decreased from 1.04 to 0.65 mg/dL at 44 hours. Laboratory tests for liver function showed increases in SGOT, SGPT, LDH and ALP immediately after the operation. They decreased during the preservation period and tended to increase again after 36 hours in the study group. The pancreas and duodenum had minimal changes during the preservation period. Secretions from these organs ranged from 1.4-8.9 ml/hour (total 60-500 ml). Blood amylase levels maintained stable during the preservation period. Total urine output ranged from 1600 to 2500 ml during the preservation period, (28-60 ml/hour) in the study groups. No premature renal failure occurred in the study group. RBC concentrations were maintained stable during preservation by blood or plasma transfusions. Plasma infusion was always necessary in all experiments to keep hematocrit at normal levels. WBC counts had a continuous decrease during the preservation period in both groups ($p < 0.0001$ at 44 hours). Blood platelet levels had slight decrease during the preservation period ($p < 0.01$ at 40 hours). Serum potassium, calcium and glucose were replaced as needed to maintain normal levels. Serum potassium tended to decrease due to high volume urine output. However, a relatively lower serum potassium did not cause much trouble. Sodium and chloride levels remained in the normal range during the preservation period in both groups. Unlike the control study, no severe liver congestion occurred or premature renal failure occurred and lung function was good up to more than 55 hours in the longest survival experiment. These studies clearly indicated that DADLE was comparable to HIT in effectively extending organ survival time of the multiorgan autoperfusion system.

In a preliminary study using HIT, one lung transplantation was performed after 33 hours of preservation. A dog weighing 23 kg of body weight which matched with the donor was used as recipient. The left lung was removed and the left lung from a donor organ system which had been preserved for 33 hours was substituted in a standard fashion (Veith *et al.*³⁷). The right pulmonary artery was ligated immediately after the transplantation, leaving the transplanted left lung responsible for whole body oxygenation. The animal was watched for 12 hours. Lung function was very good during this period and blood pressure was stable. No pulmonary edema was evident. When 100% oxygen was utilized, PaO_2 reached as high as 450 mmHg (Fig. 26). At the end of the observation, the left lung appeared normal with only a few red spots present.

The effect of HIT on platelet aggregation was studied in one dog during the preservation protocol. Platelets behaved normally prior to HIT administration, with a dose-response relationship between the amount of adenosine diphosphate (ADP) added and the extent of aggregation. However, after HIT infusion, even though the platelets aggregated normally in response to ADP, the platelets disaggregated shortly after ADP stimulated aggregation, despite the use of high doses of ADP, as shown in the Fig. 27. A similar experiment was performed in another dog study. The particles were induced by the trauma of a laparotomy. The blood in the inferior vena cava was imaged by high resolution ultrasound before, and at 20 and 60 minutes after drug administration. A proven HIT emulating drug, the delta opioid, DADLE, was infused intravenously at a concentration of 1 mg/kg. The particles were counted,

sized and their brightness determined. The product of these numbers is termed the "aggregate score." The aggregate score was 66,764 before DADLE infusion, and fell to 9,847 20 minutes after DADLE infusion which appeared to represent the peak effect. The aggregate score was 30,968 at 60 minutes after DADLE infusion.

DISCUSSION

The complete chemical and pharmacological characterization and ultimately localization of a molecule(s) which can induce or "trigger" natural mammalian hibernation and a comparable state in non-hibernating primates may have far reaching implications. Besides providing a more comprehensive understanding of the biochemical basis for this remarkable physiological phenomenon, it would afford us an opportunity to explore the clinical and pharmacological potential of this molecule in a variety of non-hibernating recipients.

The relatively slow progress in defining the chemical identity of the HIT molecule(s) may primarily be attributed to the necessity of utilizing a bioassay requiring induction of hibernation in summer-active ground squirrels (a very restrictive seasonal time frame for testing). We have now developed a rapid in vitro cell culture bioassay system utilizing three criteria (DNA synthesis inhibition, protein synthesis inhibition and intracellular calcium release) which can be utilized year round for demonstrating HIT-activity of more highly resolved plasma fractions. Moreover, this bioassay system may ultimately be utilized for determining the molecular mechanism(s) by which HIT and the delta opioid, DADLE, act at the cellular level.

Our primate experiments have clearly demonstrated that hibernators are not unique in their ability to respond dramatically to the introduction of the opioid-like HIT molecule and indicated that the clinical potential for such a profound metabolic inhibitor and its related compounds may be vast. It therefore seemed reasonable for us to investigate whether the HIT molecule and/or DADLE, could effectively extend tissue survival time in a multiorgan autoperfusion system developed by Chien et al.^{21,22} The multiorgan autoperfusion technique has the following advantages: 1. It has no ischemic time from removing the organs to preserving them in vitro. This is especially important for such organs as the heart, lungs and liver because they are very vulnerable to ischemia. 2. It retains a natural circulation. A circulatory volume is large enough so that no foreign material is necessary to help the circulation. As a result: A. No anticoagulation (heparin) is necessary. B. Blood circulates in a natural vascular tree so that hemolysis is minimal. 3. It is a self-contained organ block with a relatively complete physiologic environment. Metabolic wastes are removed and water and electrolyte balance is maintained automatically by the kidneys and major metabolic processes are taken care of by the liver. 4. The preparation is relatively simple. A respirator is the only necessary commercial equipment. 5. Because of the organs are outside the body, physiologic, pharmacologic and pathologic studies are convenient.

Our preliminary studies utilizing HIT and DADLE for extending survival time of a multiorgan autoperfusion system have provided basic insight into the mechanisms by which the opioid-like HIT molecule and DADLE act at the tissue level (i.e. do these molecules act by depressing metabolism of the multiorgan preparation or by maintaining or improving perfusion to the isolated organs by preventing aggregation of circulating cellular components).

In these studies, liver congestion, pulmonary edema, and premature renal failure were major features before using HIT or DADLE, severe liver congestion always occurred along with

deterioration of the heart, lungs, liver and kidney. Swelling of the canine liver can be produced by high perfusion pressure, trauma to the organ, anaphylactic shock, peptone shock, or an injection of histamine. This swelling is thought to be due to an obstruction of the outflow of blood from the liver as a result of contraction of hepatic venous sphincters. Such sphincters are known to be present in several animal species, including the dog, and there is some evidence of their presence in human (Gibson³⁸). This mechanism may play a role in the physiologic response when increased circulatory demands are associated with conditions of stress. The exact site of the sphincter mechanism is still not clear. A strong contraction of the hepatic vein was observed at its junction with the inferior vena cava. A large dose of epinephrine administered prior to the injection of contrast medium could prevent contraction of the hepatic vein sphincters. Conversely, the same dose of epinephrine given after the sphincter had contracted failed to inhibit the already contracted sphincters (Walker *et al.*³⁹). Our preliminary findings suggested that the infusion of HIT and DADLE released already contracted sphincters quickly, eliminated liver congestion, reduced edematous changes in the heart, kidney, and reduced pulmonary edema, along with the improvement of heart, lung, liver and kidney functions. In preliminary microcirculation studies which have just begun injection of HIT-containing plasma in rat resulted in increased microvascular diameter along with increased blood flow in microvascular system in cremaster muscle. These results have suggested that HIT can improve blood circulation via vasodilation in both macrovascular and microvascular levels.

In summary, the main effects of HIT or DADLE in our organ preservation study included: substantial extension of tissue survival time (up to 3 times of the control group), elimination or reduction of hepatic congestion. Improvement of renal function, reduction of pulmonary edema, and reversal of platelet aggregation. Further study on the mechanism(s) by which HIT or DADLE increased tissue survival time revealed three possible beneficial effects of HIT and DADLE: 1) they reduced tissue metabolism, 2) they reduced or eliminated platelet and/or leucocyte aggregation, and 3) they improved microcirculation via vasodilation. The superior effects of HIT and DADLE make us to believe that there might be some very important factors in the winter hibernator's plasma and DADLE. If these effects can be duplicated and confirmed, it is possible that in the future HIT or DADLE could have important usage in the treatment of trauma, microvascular disease, multiple organ failure, and tissue preservation.

TABLE 1. INHIBITION OF DNA SYNTHESIS BY AFFIGEL-BLUE COLUMN FRACTIONS^a

Frac. ^e	% Inhibition ^b					
	TRMP wpcf ^c	cells spf ^d	CREF wpcf	cells spf	SB3 wpcf	cells spf
0	100	0	100	7	100	0
1	4	0	39	27	95	16
2	48	18	0	52	0	0
3	100	30	100	35	100	5

a Serum starved cells were stimulated with 5% FBS in the presence of 7.5% plasma or concentrated fractions from the Affigel-Blue column.

b DNA synthesis was measured by determining ³H-thymidine incorporation during the interval of 12-18 hrs after FBS stimulation. The % inhibition was determined by comparing the amount of thymidine incorporation in the presence of the different column fractions with the amount of observed with 5% FBS alone.

c wpcf-protein fractions obtained from the winter-hibernating plasma.

d spf-protein fractions obtained from the summer-active plasma.

e The tested fractions are as follows: 0=unfractionated plasma, 1=protein that passed through the column, 2=protein that was weakly bound to the column and removed with the 0.02M phosphate buffer, 3=protein eluted with 1.4M NaCl (albumin fraction).

TABLE 2. STIMULATION OF DNA SYNTHESIS BY AFFIGEL-BLUE COLUMN FRACTIONS^a

Frac. ^e	% Stimulation			
	TRMP wpcf ^c	cells spf ^d	CREF wpcf	cells spf
0	133	108	127	112
1	105	30	17	1
2	78	0	17	1
3	94	83	95	75

a Serum starved cells were stimulated with 0.5% plasma or concentrated plasma protein fractions

b DNA synthesis was measured by determining ³H-thymidine incorporation at 12-18 hours after stimulation of the serum starved cells. The % stimulation was determined relative to stimulation with 5% FBS.

c wpcf-protein fractions obtained from winter-hibernating plasma

d spf-protein fractions obtained from summer-active plasma

e Tested fractions are same as in Table 1

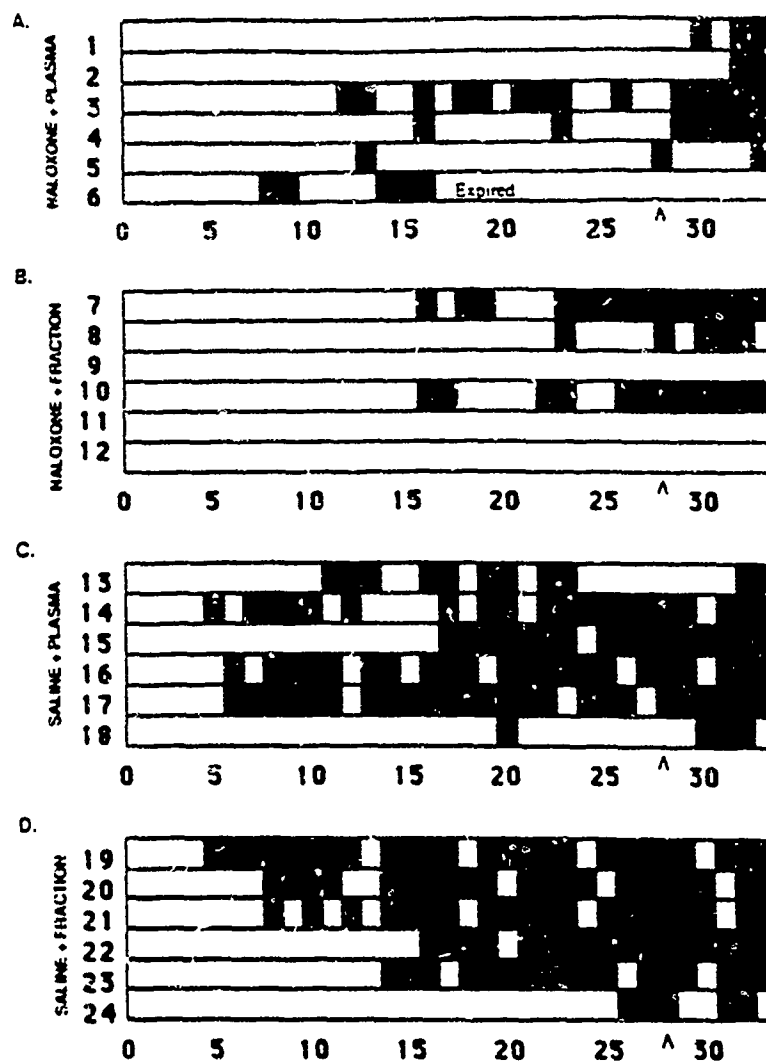


Figure 1. Patterns of Summer Hibernation In *Citellus tridecemlineatus* following Injections. The black bars indicate days spent in hibernation. Arrows at day 28 after pump implantation show when pumps should become empty. Naloxone Pump Squirrels groups A & B.

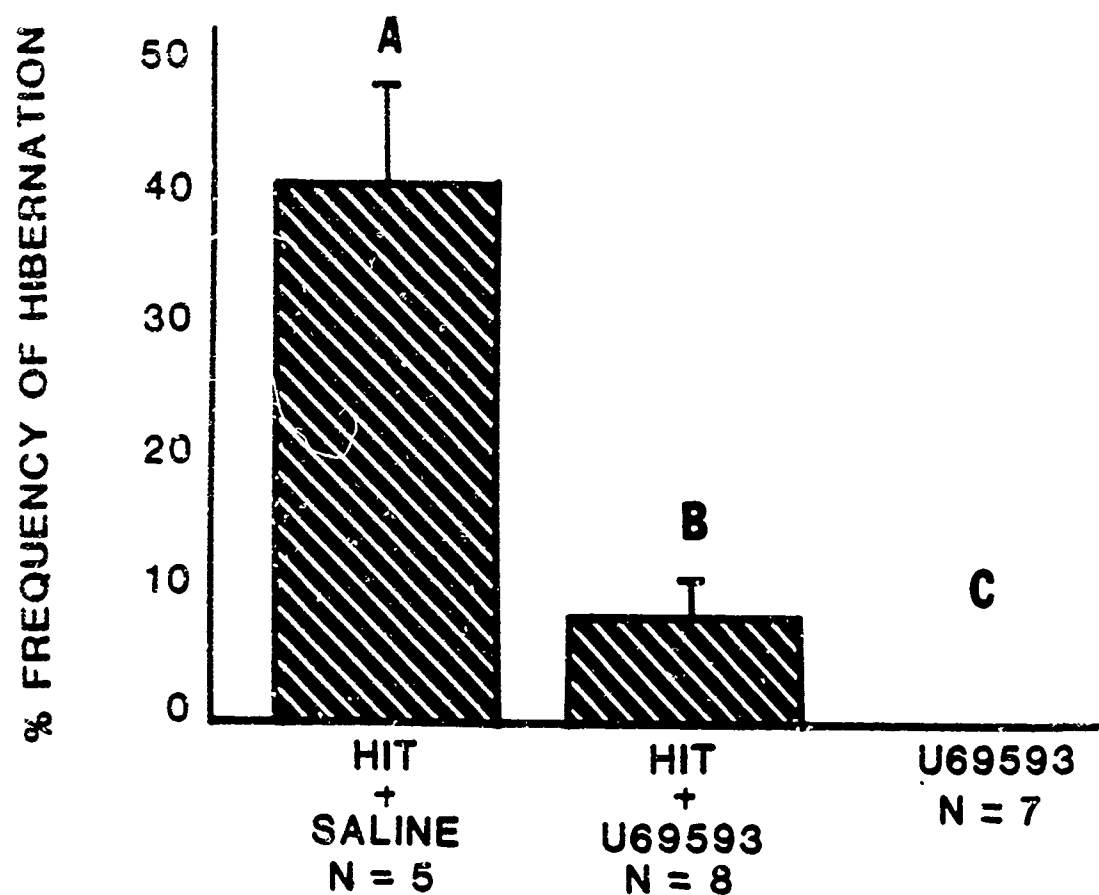


Figure 2. Percent frequency of hibernation (days spent in hibernation versus total observation days) induced by HIT. HIT combined with U69593 and U69593 alone in summer-active ground squirrels. Opioids were delivered via osmotic minipumps.

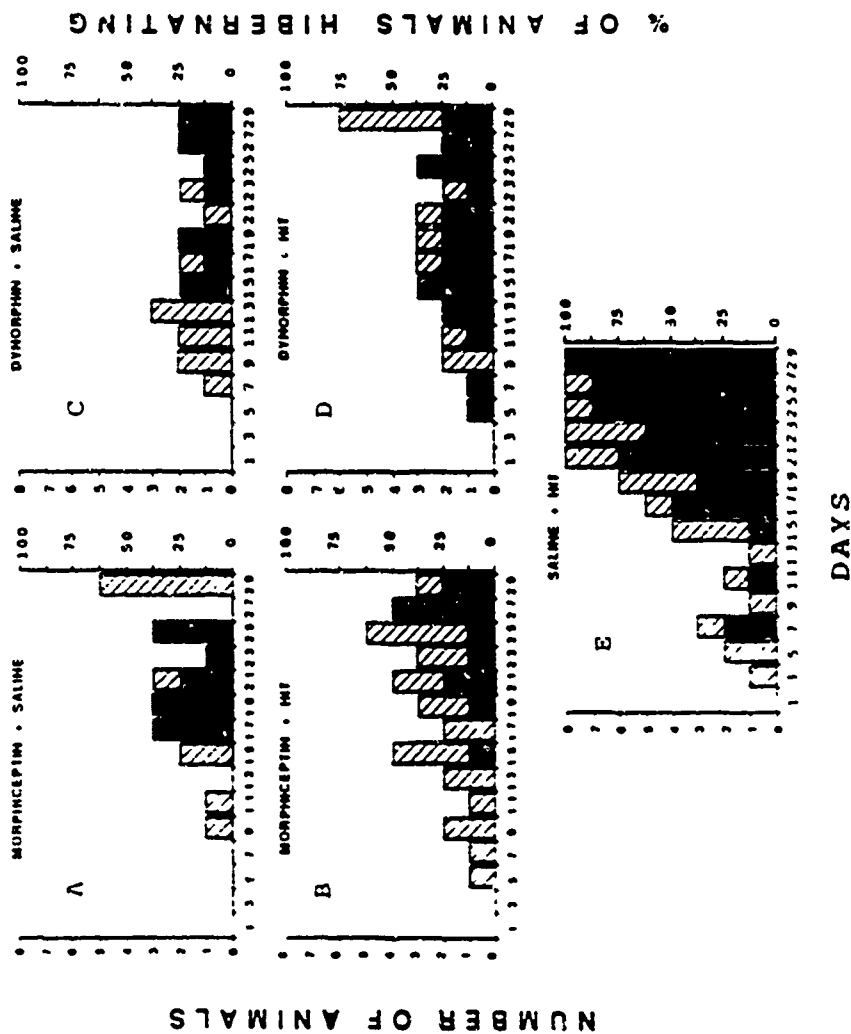


Figure 3. Effects on summer-active ground squirrels of morphine, morphine, and saline in the osmotic pumps and saline injection is presented in Fig. 2A, while that of morphine, morphine, and HIT injection is presented in Fig. 2B. Similarly, the effects of morphine in the osmotic pumps and saline injection is presented in Fig. 2C, while that of morphine in the osmotic pumps and HIT injection is presented in Fig. 2D. For comparison purposes, Fig. 2E depicts the effects of saline in the osmotic pumps and HIT injection. The number of animals in hibernation on a particular observation day is indicated by black bars. Shaded bars indicated number of animals in lethargic state (responded to light and sounds stimuli) and not in hibernation. Note that whenever a shaded bar appears on top of the black bar, the actual length of the shaded bar represents number of lethargic animals. For example, in panel C, on day 17 only one animal was lethargic and four of them were hibernating. The labels on the right hand of the figure (% OF ANIMALS HIBERNATING) therefore apply to only the bars.

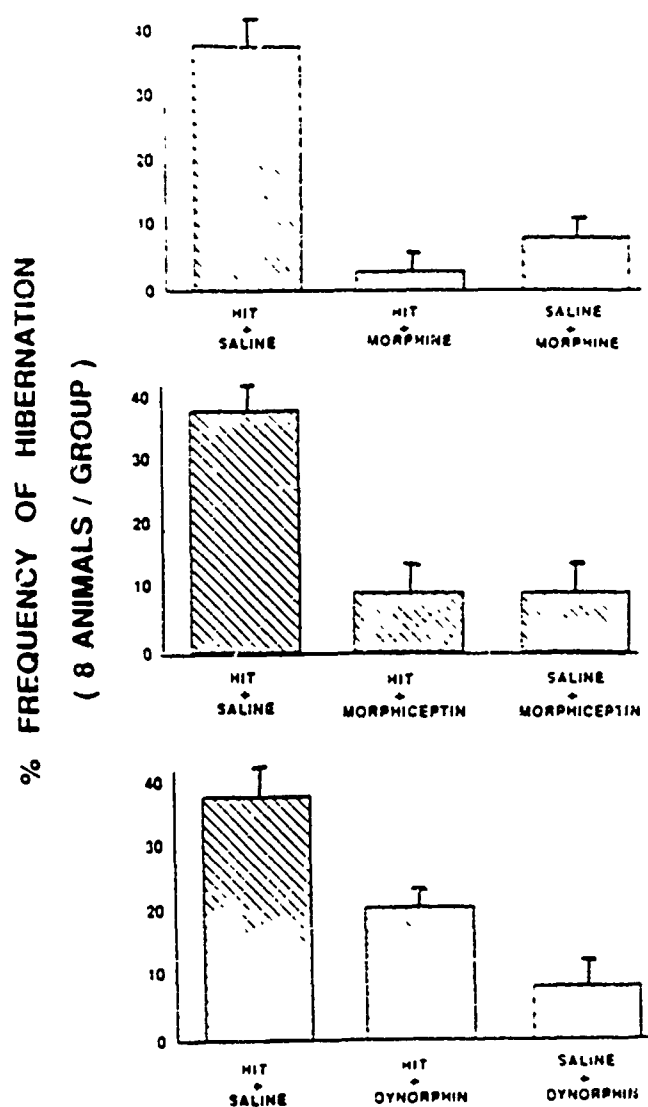


Figure 4. Percent frequency of hibernation (days spent in hibernation versus total observation days) induced by HIT, HIT combined with opioids, and the opioids alone. The % frequency of hibernation of the morphine + saline group was $7.5 \pm 2.4\%$. The morphine + HIT group had a % frequency of hibernation of $0.83 \pm 0.83\%$. The % frequency of hibernation for the morphine + saline group and the morphiceptin + HIT groups were each $10.0 \pm 2.7\%$. The dynorphin A + saline group had a % frequency of hibernation of $9.2 \pm 2.6\%$, while the dynorphin A + HIT group had a % frequency of hibernation of $16.7 \pm 3.4\%$. The % frequency of hibernation in the control group (HIT + saline) was $36.7 \pm 4.4\%$. All groups had a probability less than 0.0001.

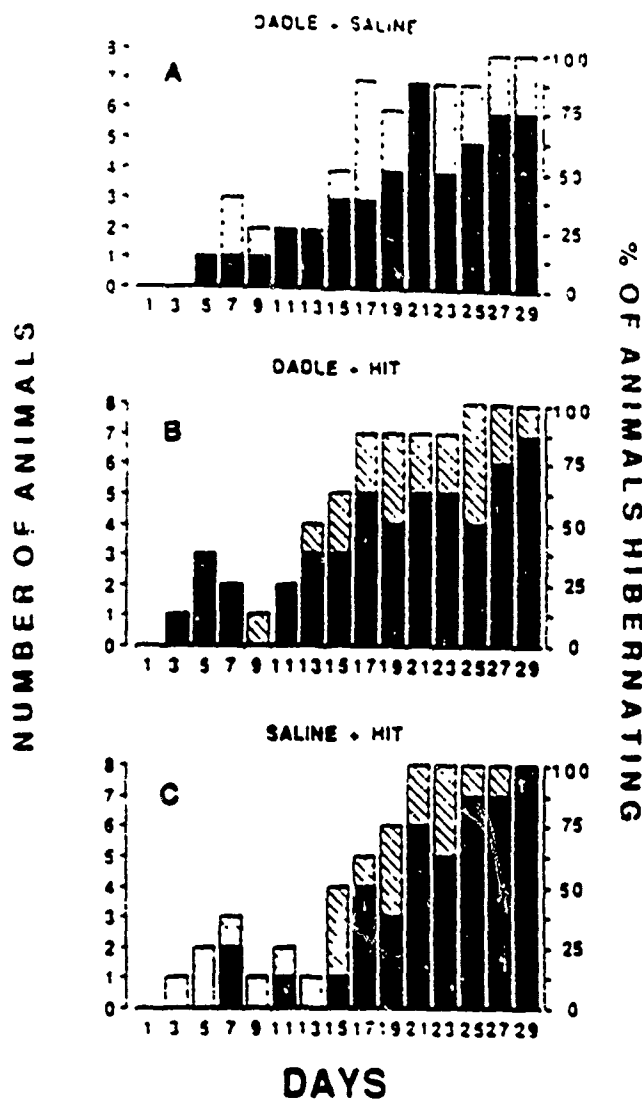


Figure 5. Effects of DADLE, and DADLE in combination with HIT injection on summer-active ground squirrels. DADLE was delivered through osmotic mini pumps at a rate of 1.50 mg/kg/day.

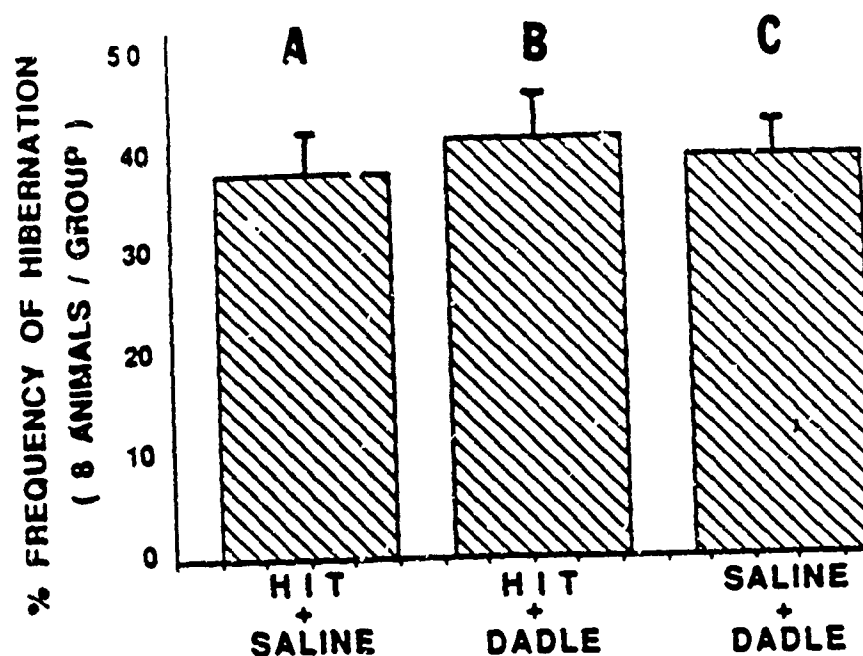


Figure 6. The percent frequency of hibernation induced by HIT plus saline (A), HIT plus DADLE (B) and saline plus DADLE (C) in summer-active ground squirrels. The % frequency of hibernation induced by DADLE + saline was $37.5 \pm 4.4\%$ with DADLE + HIT group having a slightly higher % frequency of $41.7 \pm 4.5\%$.

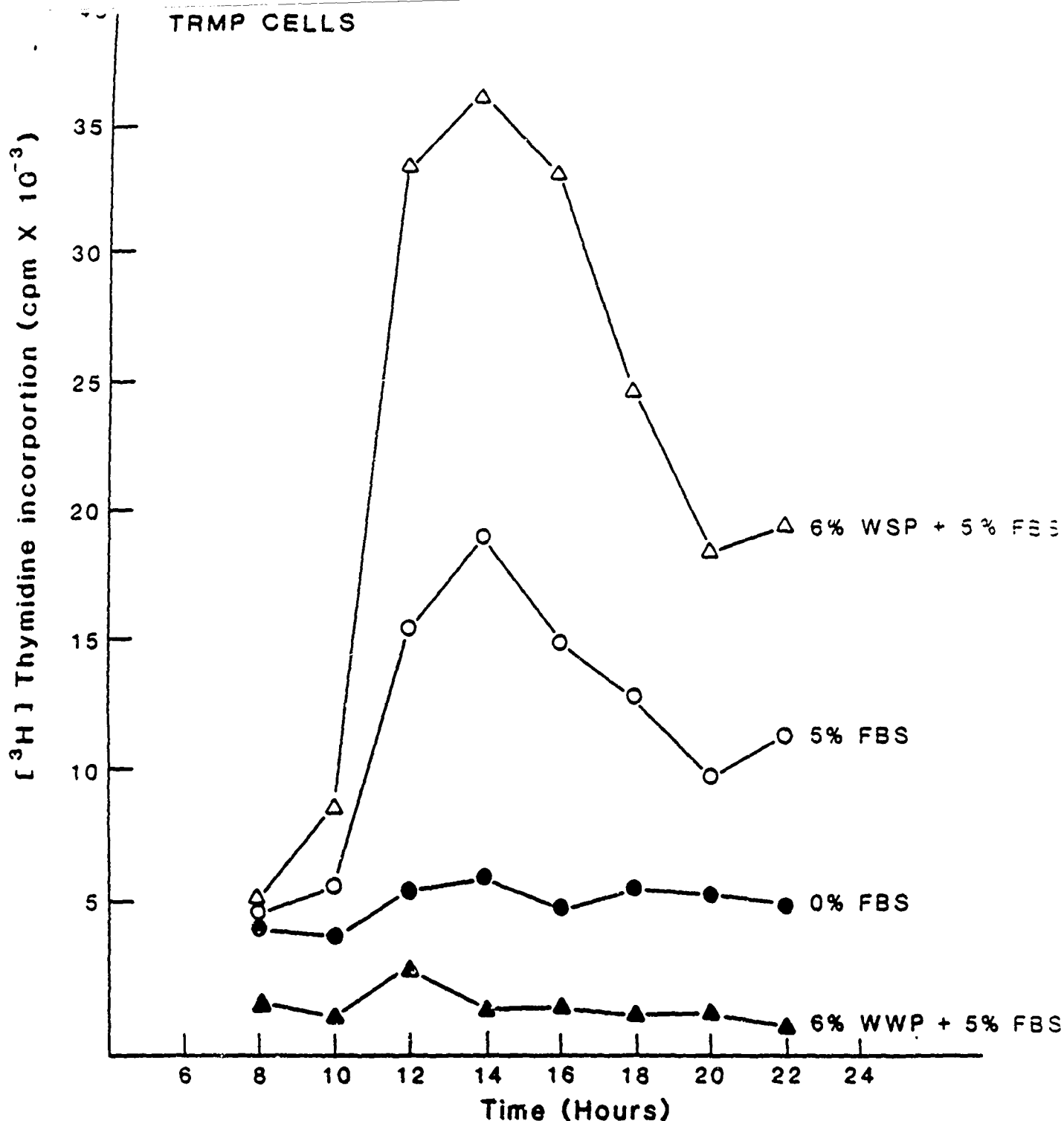


Figure 7. Plasma from winter-hibernating woodchucks inhibits fetal bovine serum (FBS) stimulation of DNA synthesis in serum starved TRMP cells. Serum starved TRMP cells stimulated with 5% FBS show increased incorporation of ³H-thymidine beginning at 10-12 h with a maximum at 16-18 h. When supplemented with 6% winter plasma (as shown) the incorporation of ³H-thymidine was inhibited, falling to a level below that seen for cells maintained in 0% FBS. However, summer plasma at the same concentration (6%) enhanced incorporation of ³H-thymidine above that seen for 5% FBS alone. Similar results were obtained for winter and summer plasma at 7.5% and 5% demonstrating the presence of an inhibitory factor in the winter woodchuck plasma.

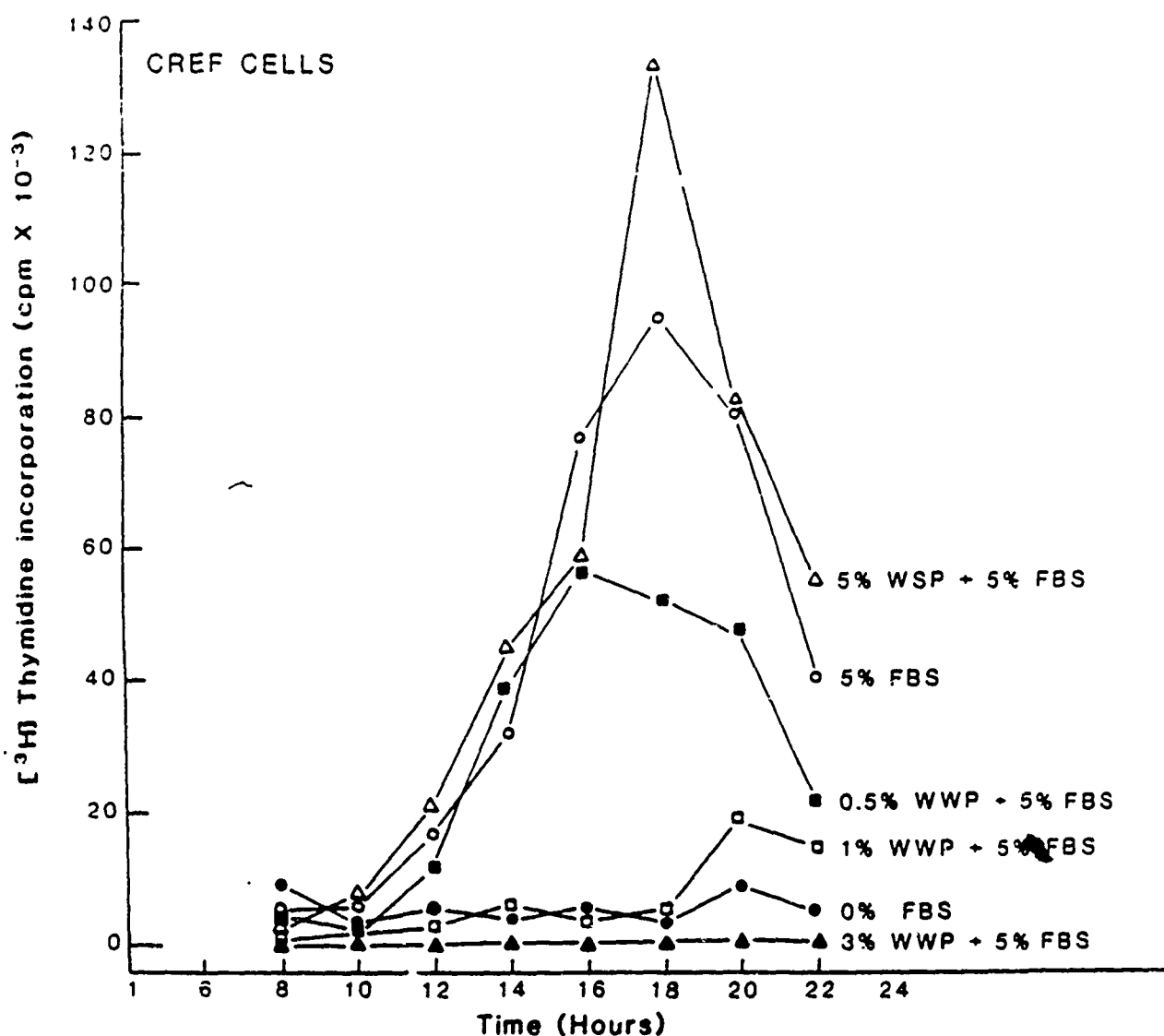


Figure 8. Complete to partial inhibition of FBS stimulated DNA synthesis in CREF cells exposed to winter woodchuck plasma. Serum starved CREF cells stimulated by 5% FBS also show increased incorporation of ^3H -thymidine beginning at 10-12h with a peak at 16-18 h. When supplemented with winter plasma at 5% or 3% (as shown) the ^3H -thymidine incorporation was totally inhibited. Partial inhibition was observed at concentrations as low as 0.5% of winter plasma. Summer plasma at 5% with 5% FBS enhanced ^3H -thymidine uptake above that observed for 5% FBS alone. This experiment demonstrated that the CREF cells were extremely sensitive to inhibition by winter woodchuck plasma.

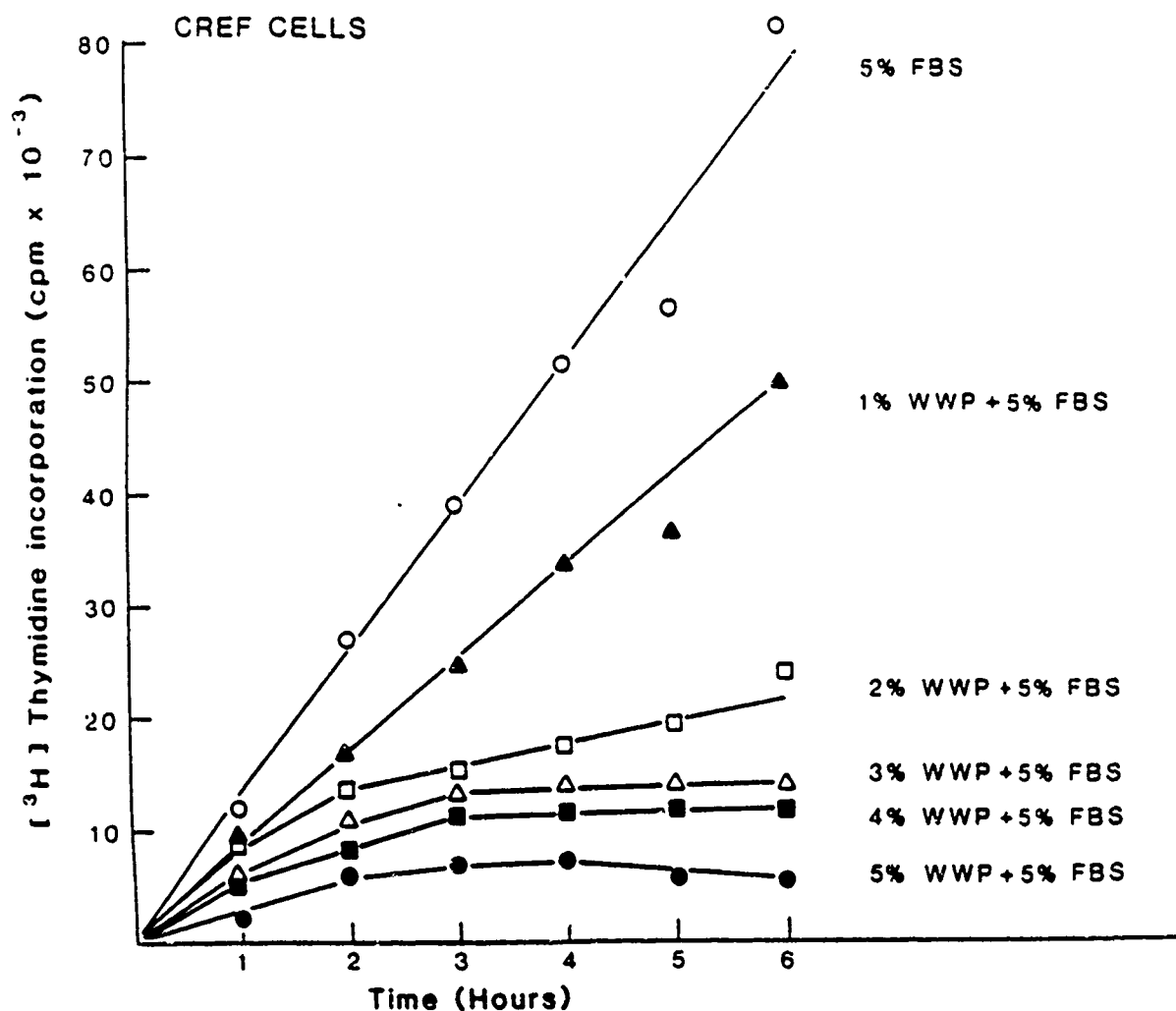


Figure 9. Plasma from winter-hibernating woodchucks inhibit DNA synthesis in continuously growing CREF cells. CREF cells growing in DMEM supplemented with 5% FBS were treated with varying concentrations of winter plasma. Significant inhibition of ^3H -thymidine incorporation was observed. The level of inhibition was directly proportional to the concentration of winter plasma added and significant inhibition was observed at concentration as low as 1%.

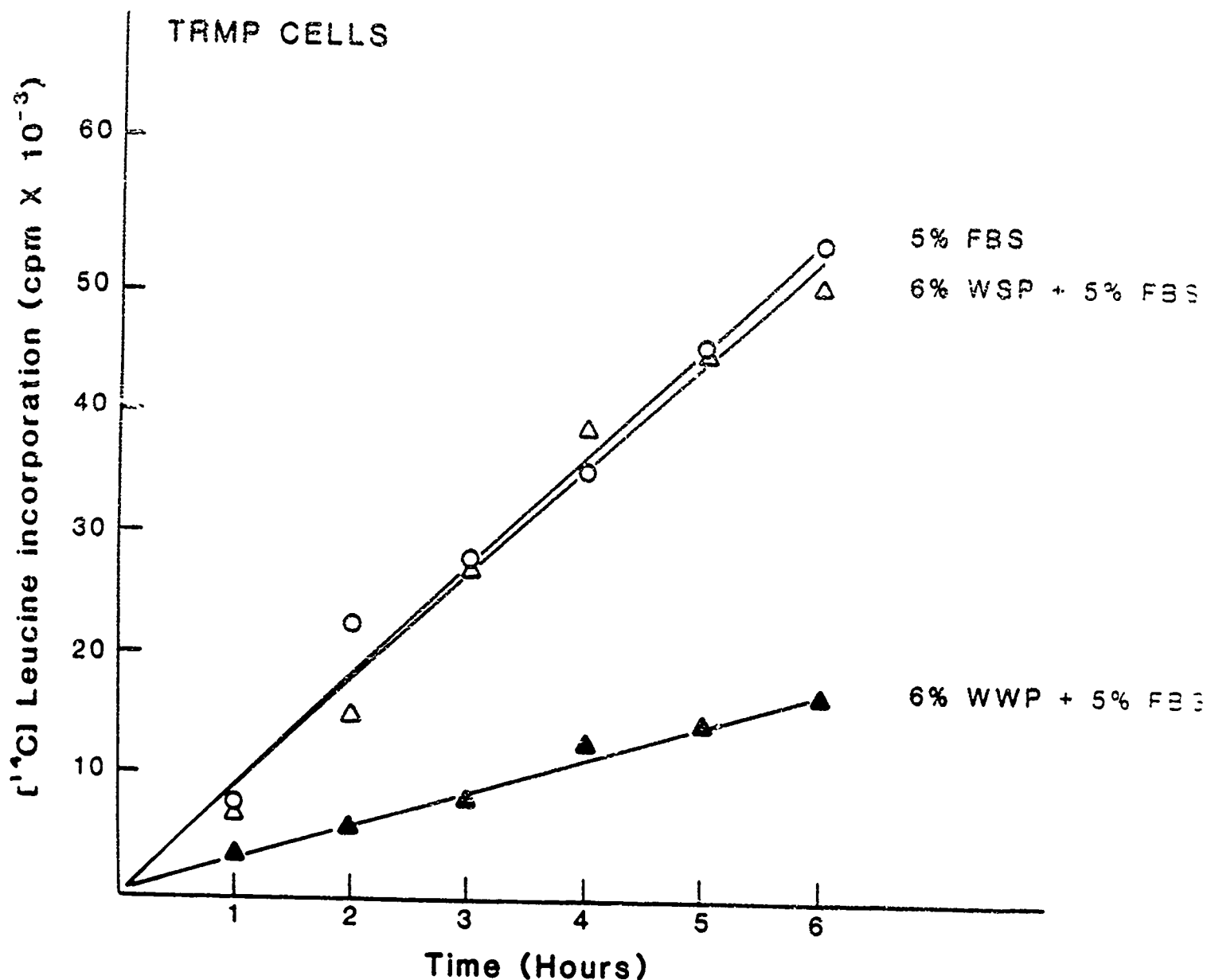


Figure 10. Plasma from winter-hibernating woodchucks inhibits protein synthesis in continuously growing TRMP cells. TRMP cells growing in DMEM supplemented with 5% FBS were treated with 6% each of summer and winter woodchuck plasma in the presence of ^{14}C -leucine. Decreased incorporation of ^{14}C -leucine was observed with the 6% winter plasma relative to that seen with both 6% summer plasma and 5% FBS or 5% FBS alone.

TRMP CELLS

FBS

- 0.5%
- ▲—▲ 0.25%
- 0.1%
- 0.01%
- 0% FBS

-32- [^3H] Thymidine incorporation (cpm $\times 10^{-3}$)

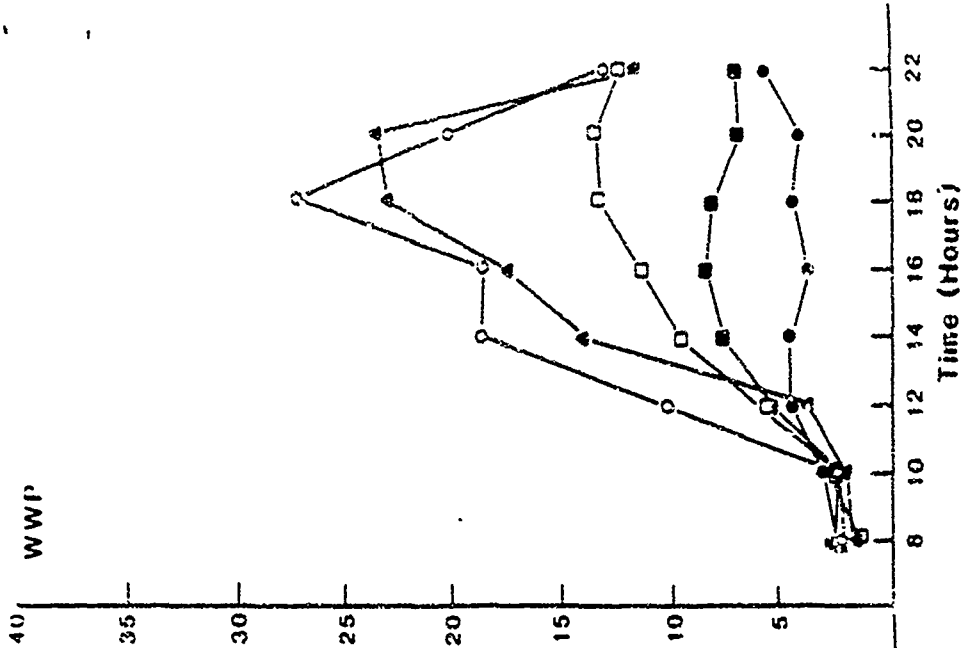
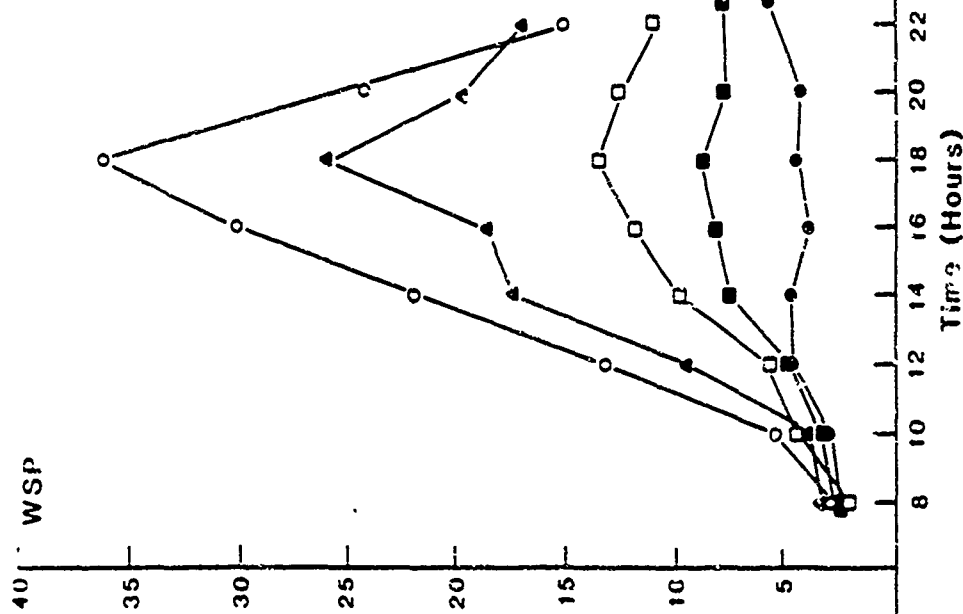
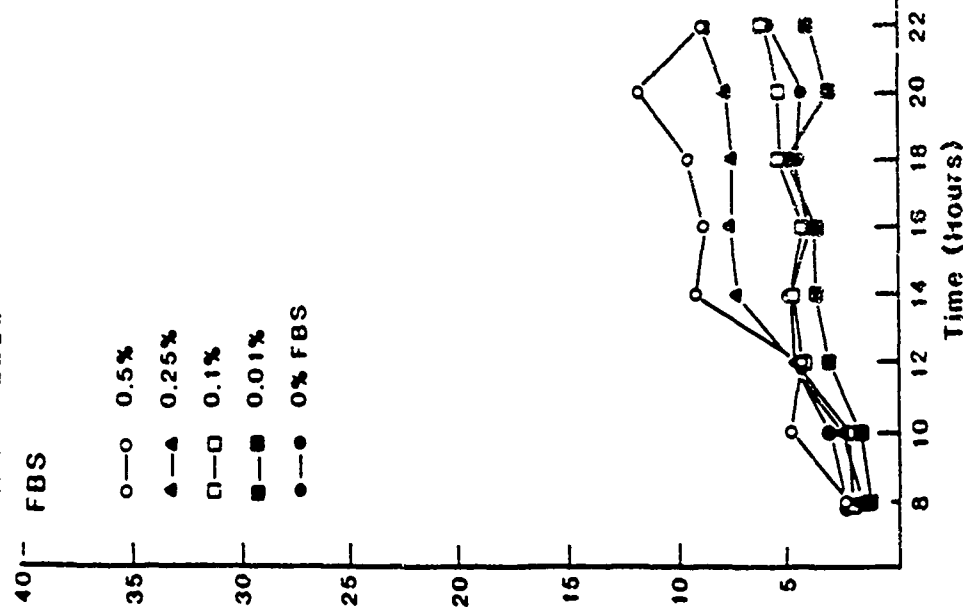


Figure 11. Low concentrations of plasma from both winter-hibernating and summer-active woodchucks stimulate DNA synthesis in serum starved TRMP cells. Serum starved TRMP cells treated with summer or winter woodchuck plasma at concentrations ranging from 0.5% - 0.01% were stimulated to incorporate ^3H -thymidine. FBS at this low concentration demonstrated only marginal stimulation of ^3H -thymidine. This would indicate the presence of a mitogen in both summer and winter woodchuck plasma. However, the mitogen in the winter plasma was revealed upon dilution.

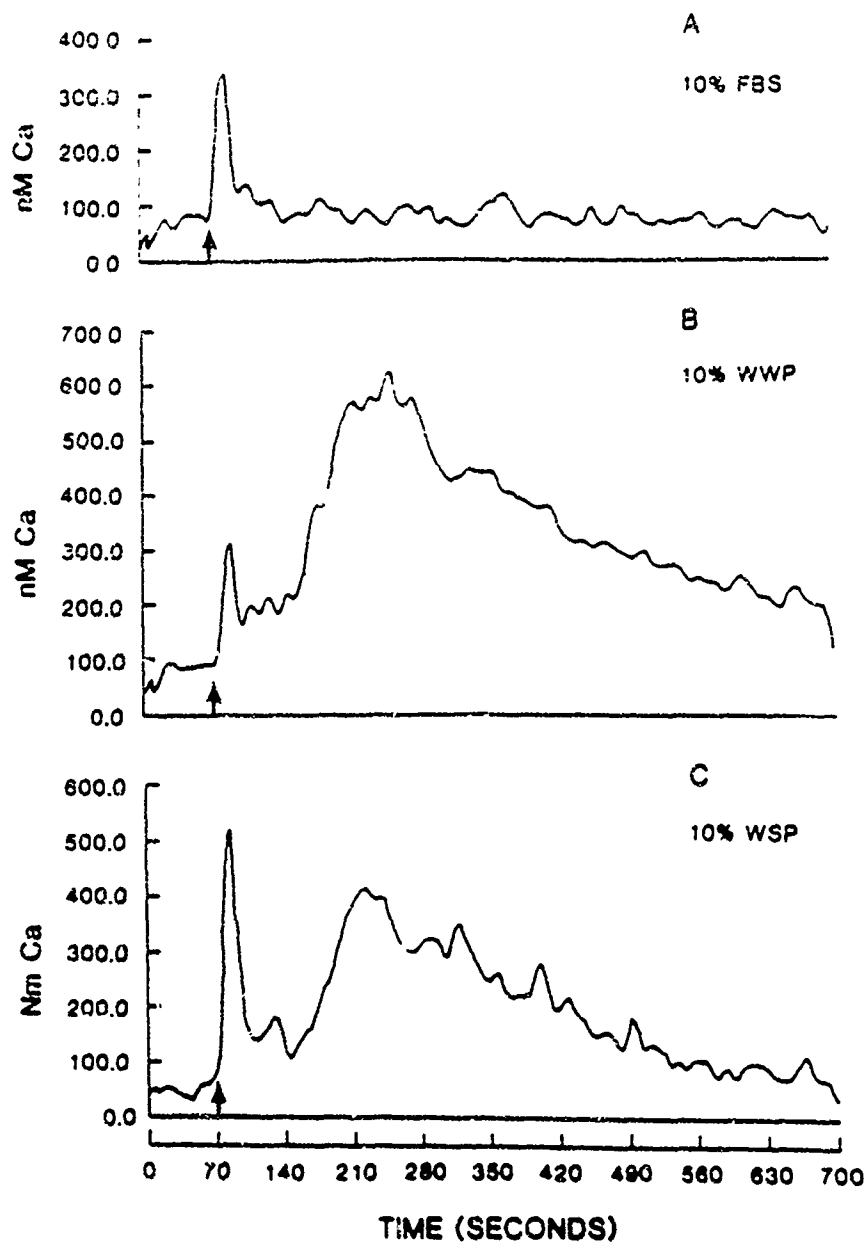


Figure 12A. Calcium response of serum starved TRMP cells exposed to 10% FBS, to (Fig 12B) 10% winter hibernating woodchucks plasma, and (Fig 12C) to 10% WSP.

MULTIORGAN AUTOPERFUSION SYSTEM

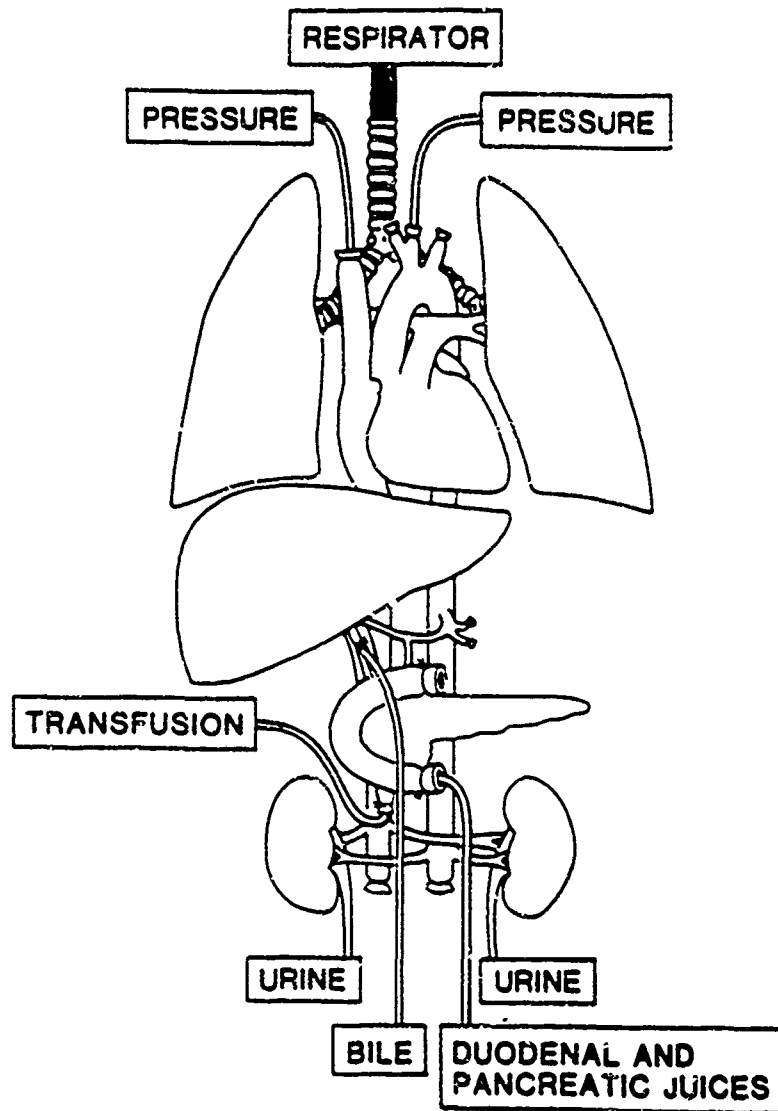


Figure 13. Simultaneous multiorgan autoperfusion preservation in which the heart, lungs, liver, pancreas, duodenum and two kidneys are preserved in one system. No foreign material is used to assist the circulation. No inotropic drugs or heparin are necessary to maintain the circulation.

SURVIVAL TIME HIT vs CONTROL

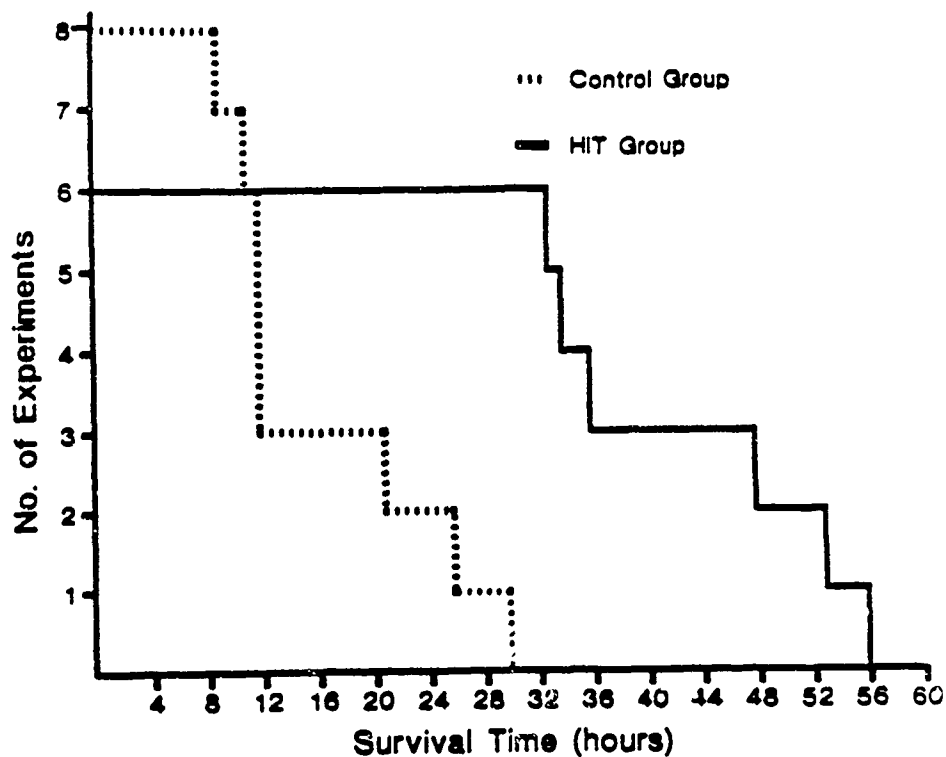


Figure 14A. Survival of individual multiorgan preparations in the control group (n=8) versus the HIT treated group (n=6).

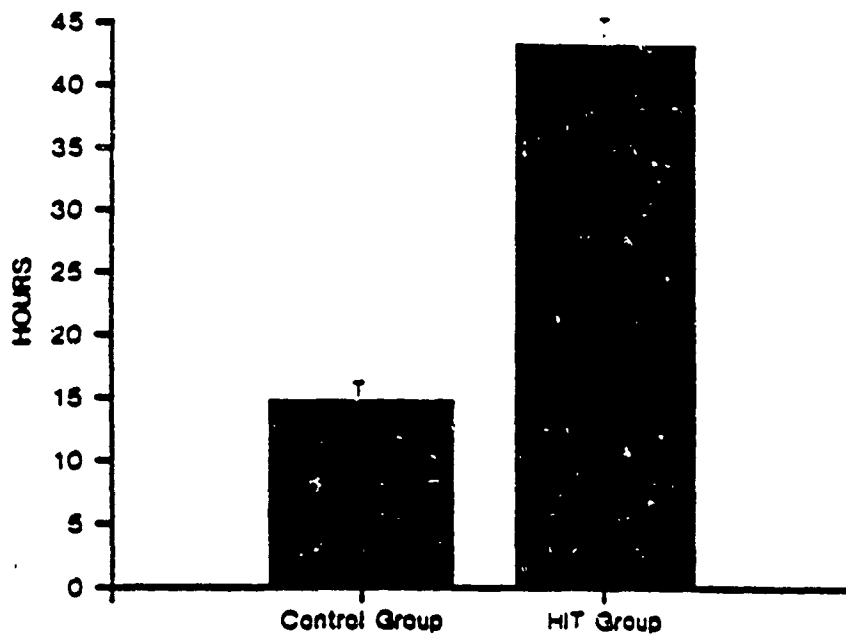


Figure 14B. Survival time in the study group with HIT and the control group without using HIT (p 0.00025). Three early deaths occurring between 33-36 hours in the study group were caused by hyperkalemia.

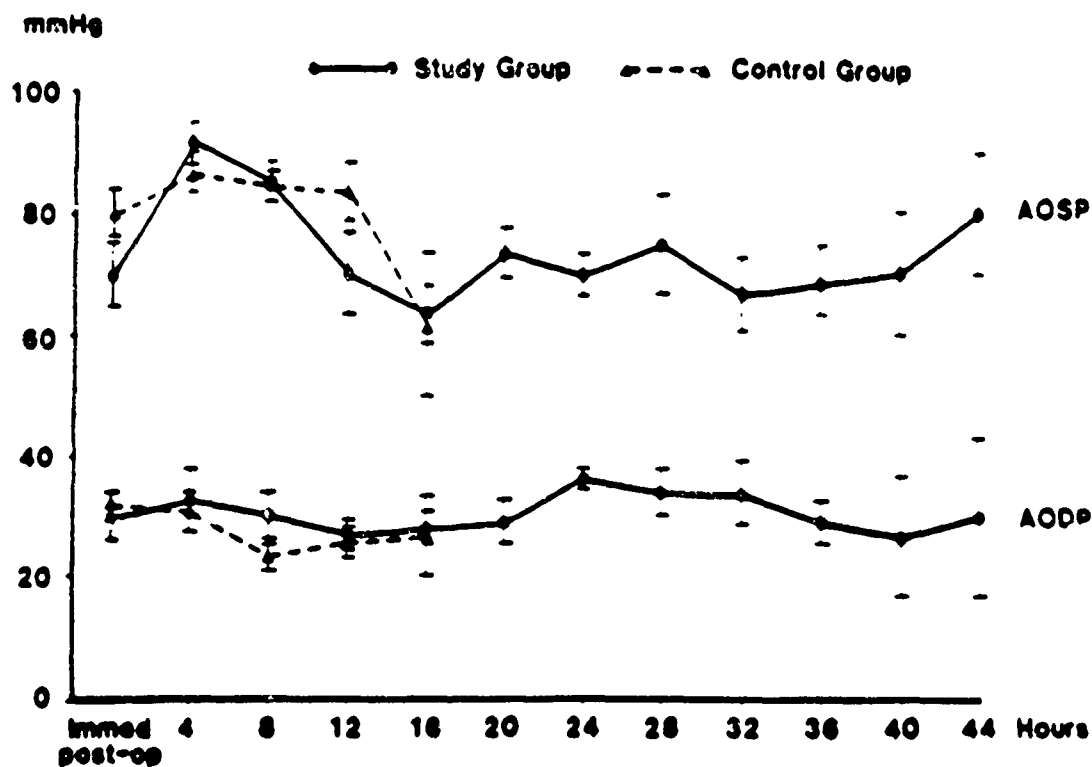


Figure 15. Aortic systolic pressure (AOSP) and diastolic pressure (AODP) during the preservation period.

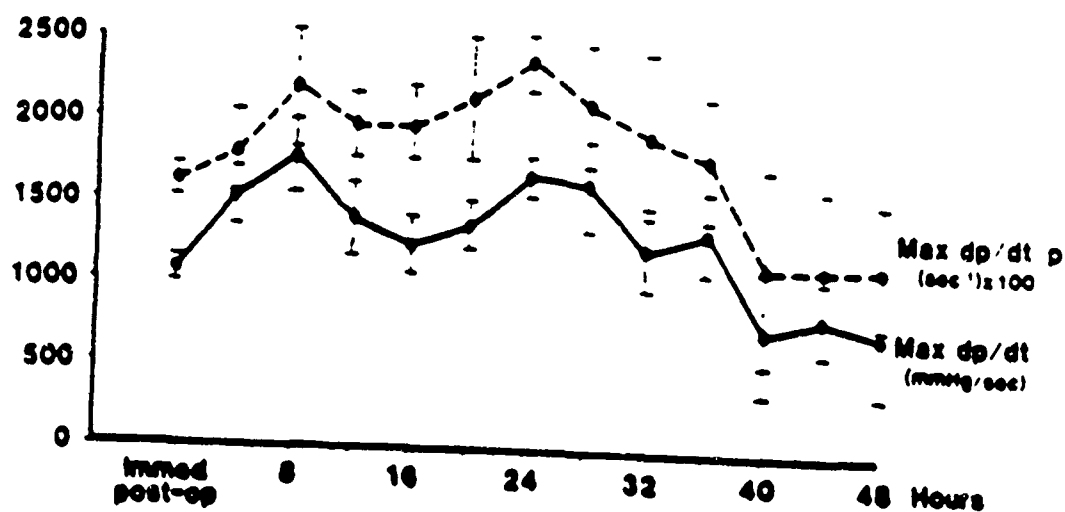


Figure 16. Changes of left ventricular maximum dp/dt and $dp/dt/p$ during the preservation period in the study group.

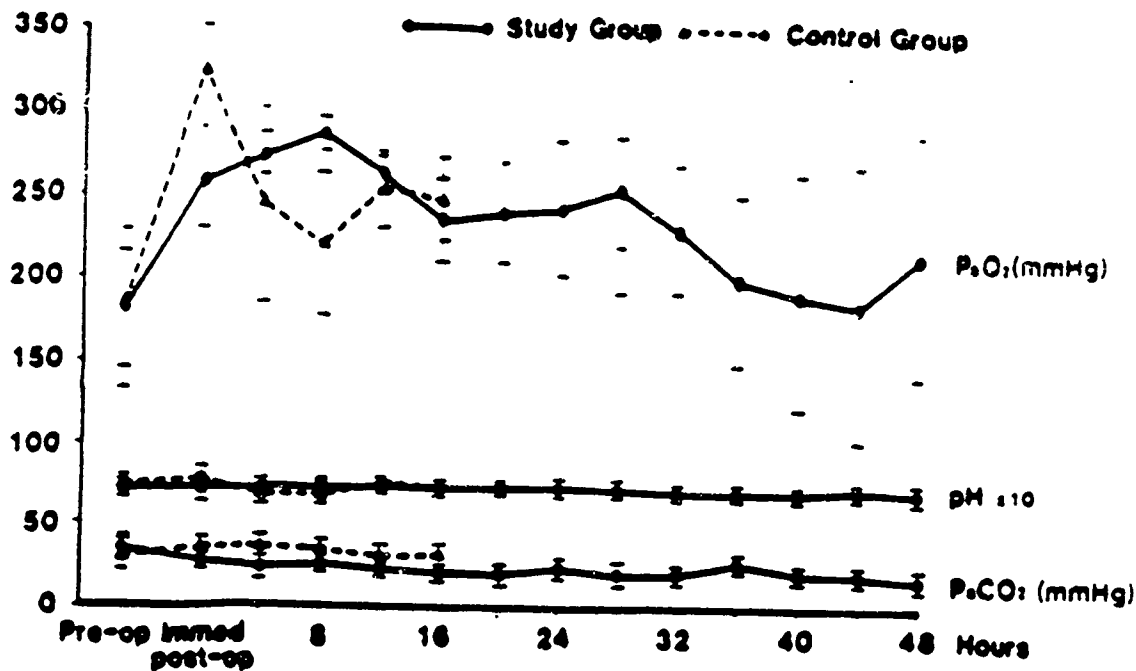


Figure 17. Change of blood gas values during the preservation period. P_{aO_2} : Arterial oxygen tension. P_{aCO_2} : Arterial carbon dioxide tension.

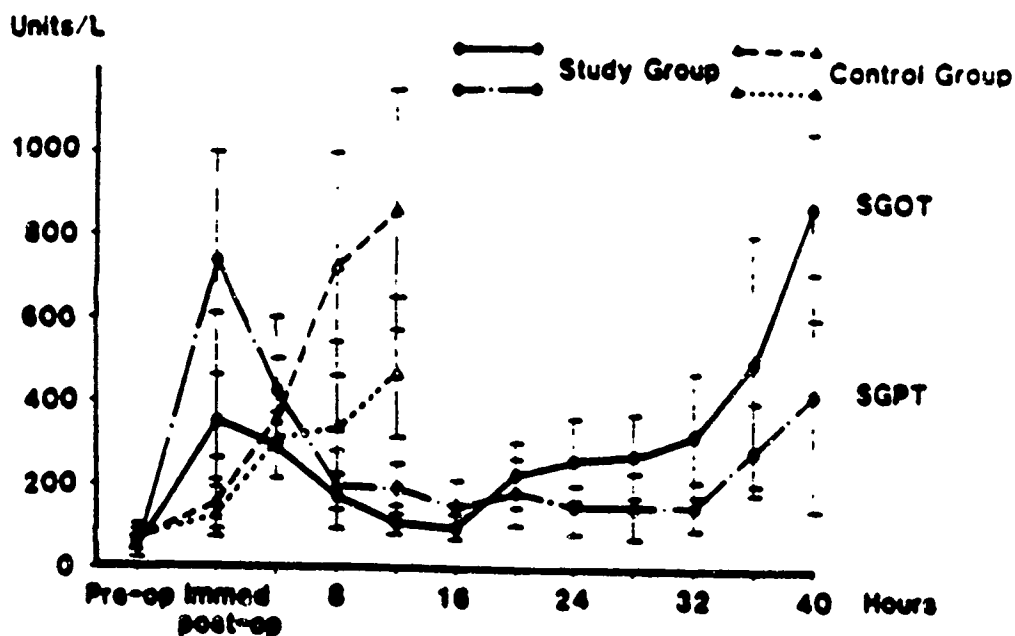


Figure 18. Serum glutamic-oxalacetic transaminase (SGOT) and glutamic-pyruvate transaminase (SGPT) during the preservation period.

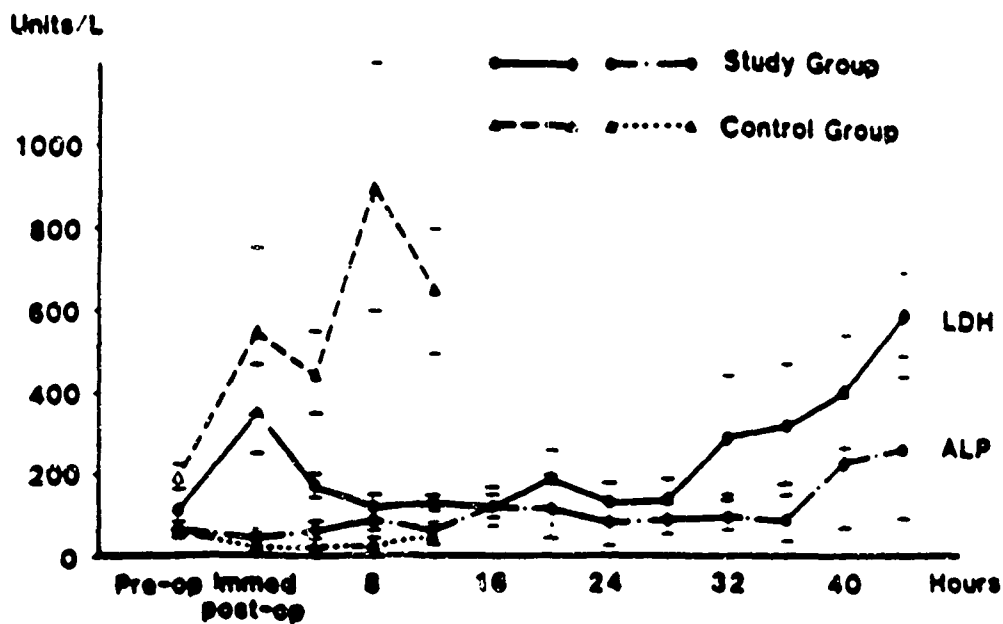


Figure 19. Serum alkaline phosphatase (ALP) and lactic dehydrogenase (LDH) during the preservation period.

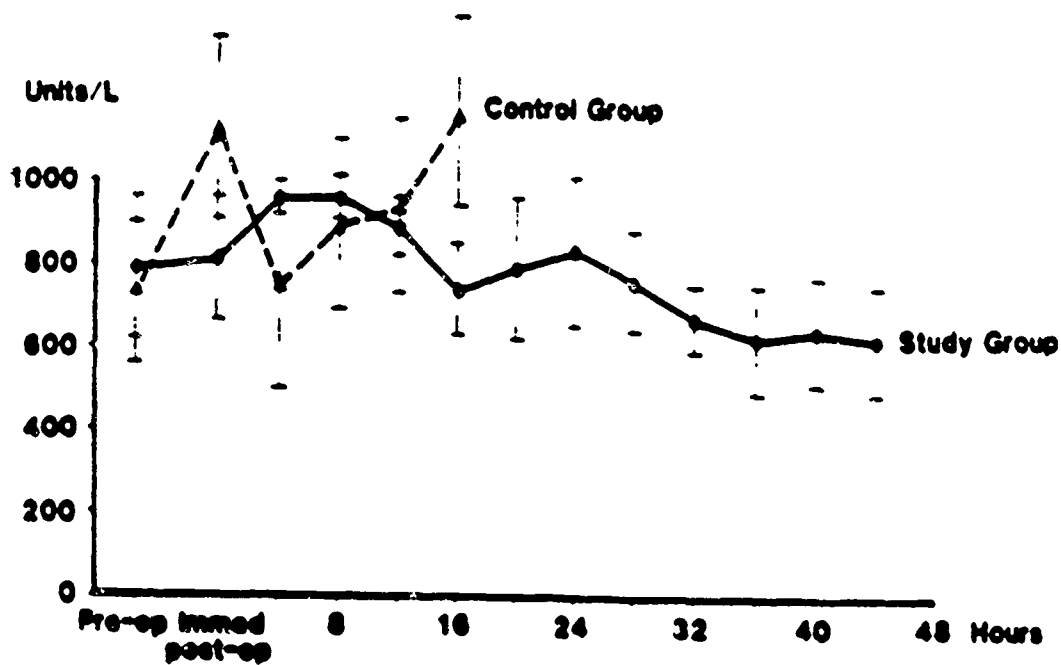


Figure 20. Changes of blood amylase levels during the preservation period.

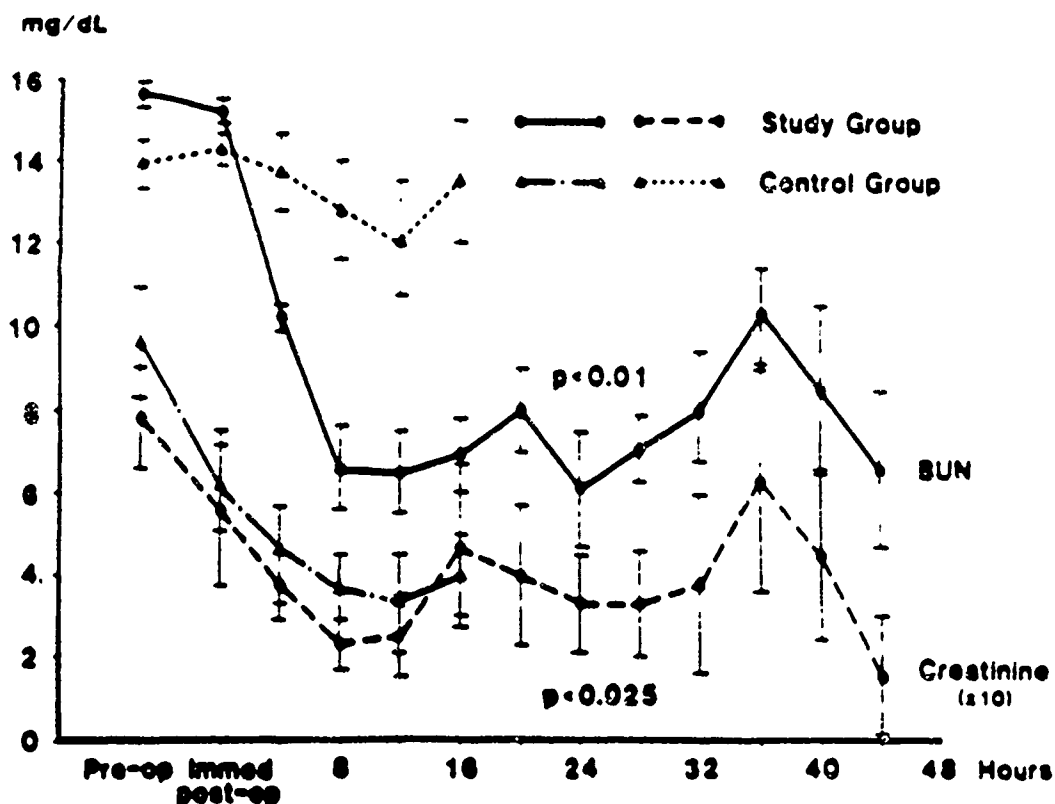


Figure 21. Changes of blood urea nitrogen (BUN) and creatinine levels during 44-hour period. (All p values are the comparisons between pre-op levels and those obtained during the preservation period).

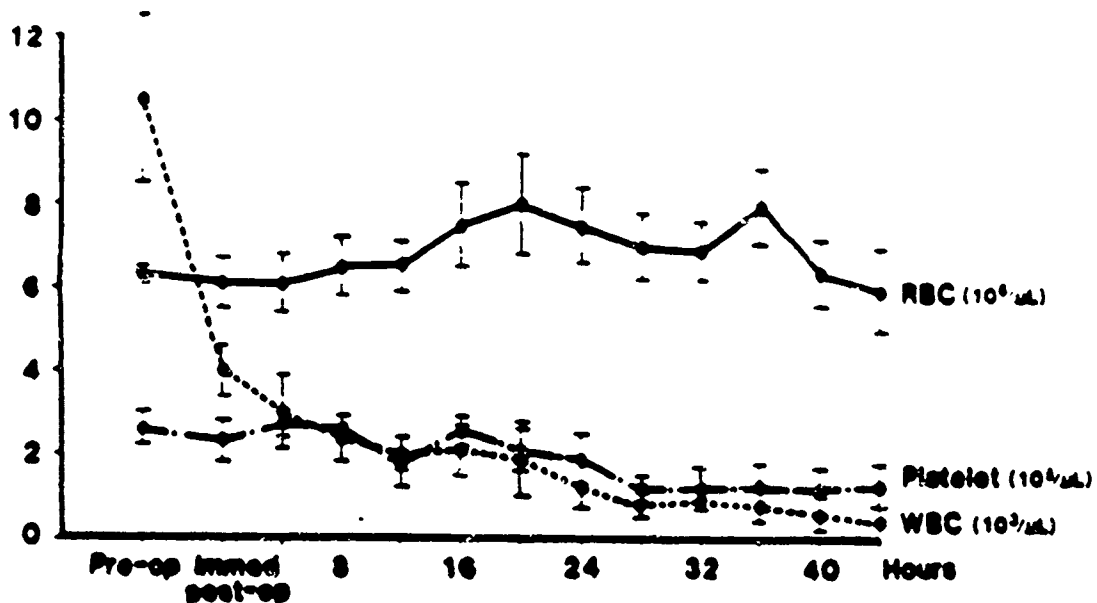


Figure 22. Changes of blood cells during preservation. RBC: red blood cell, WBC: white blood cell.

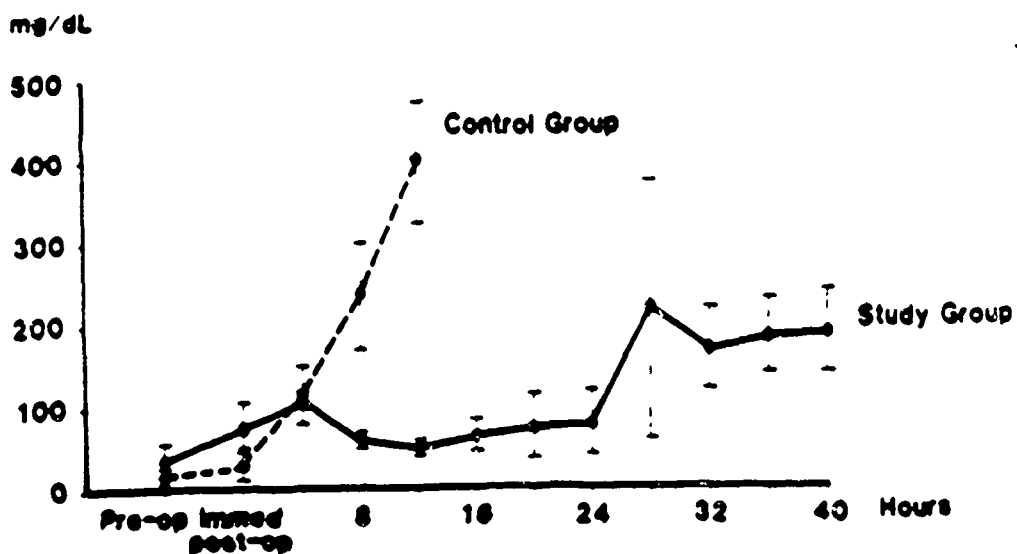


Figure 23. Change of plasma free hemoglobin during the preservation period.

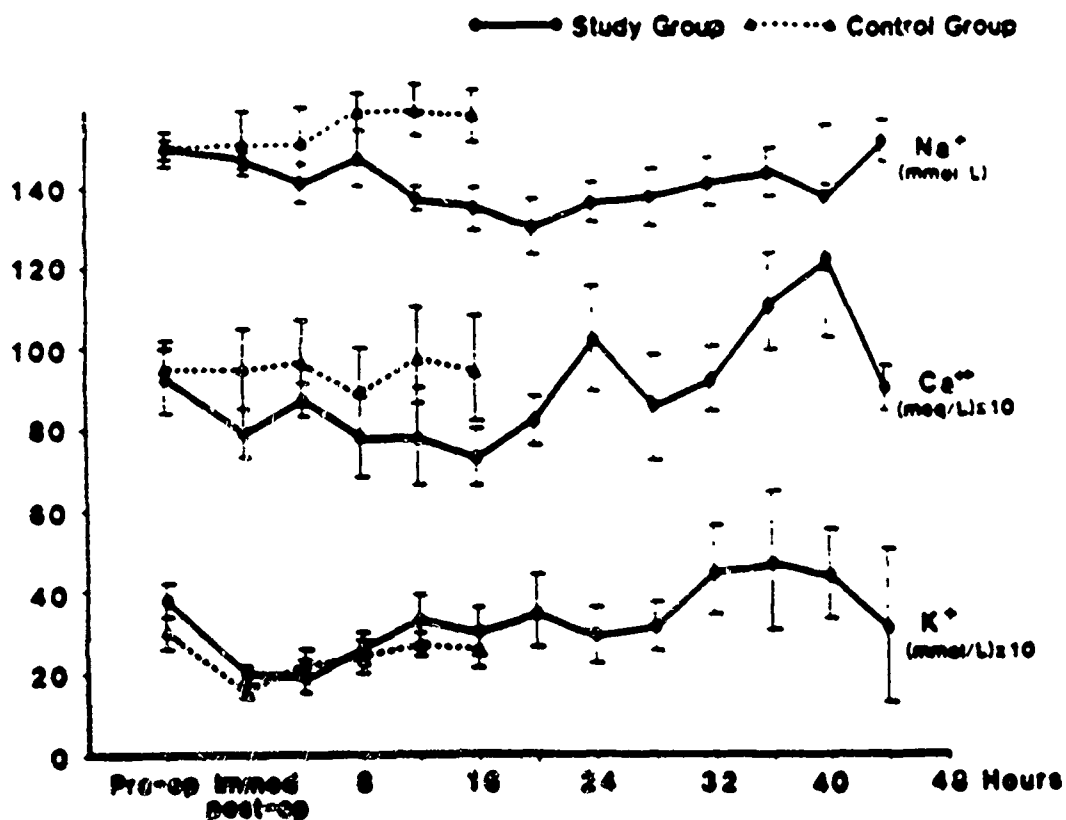


Figure 24. Changes of serum potassium (K⁺), sodium (Na⁺), and calcium (Ca⁺⁺) during preservation.

SURVIVAL TIME DADLE vs CONTROL

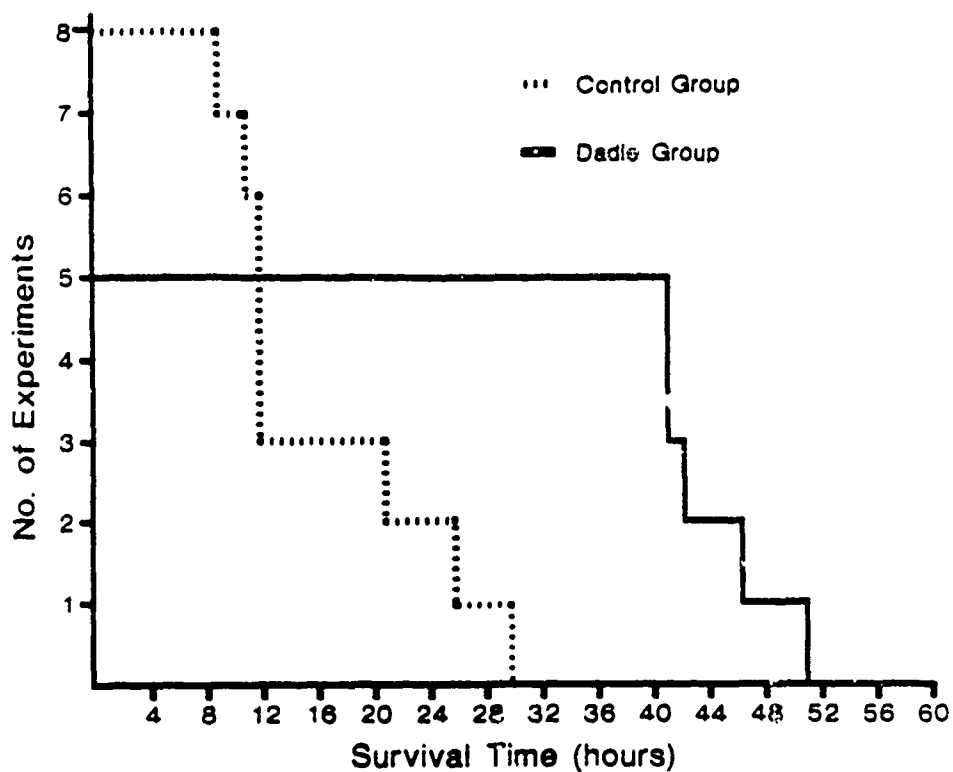


Figure 25A. Survival of individual multibacterial preparations in the control group (n=8) versus the DADLE-treated group (n=5).

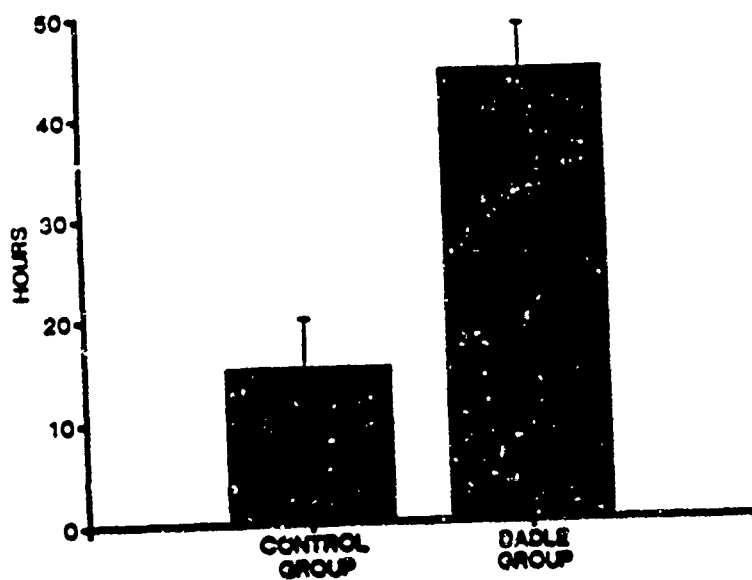


Figure 25B. Comparison of survival time between the study group using DADLE and the control group without using DADLE (p 0.0001).

LUNG FUNCTION AFTER TRANSPLANTATION

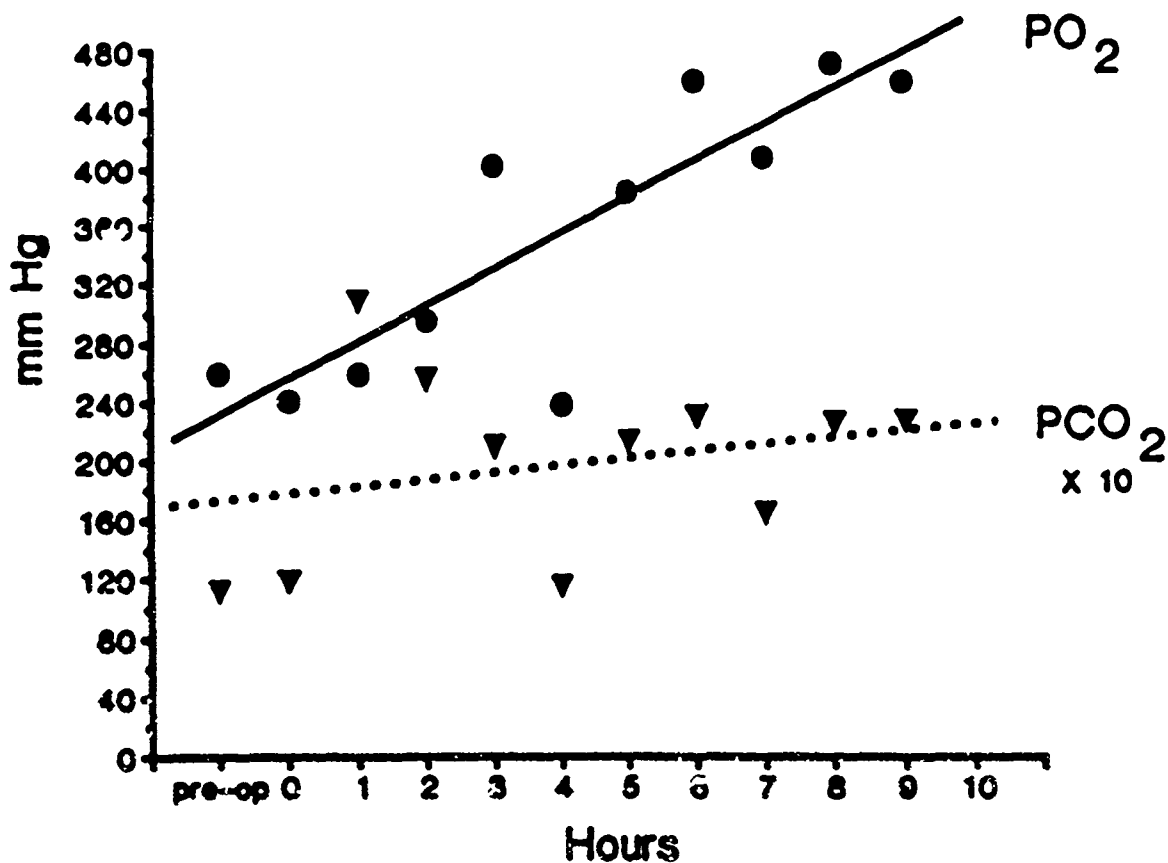


Figure 26.

Arterial blood gases of recipient dog after left lung transplantation left lung was transplanted after 33 hours of preservation in multiorgan autoperfusion system. The right pulmonary artery of remaining lung of recipient was ligated immediately after transplanted.

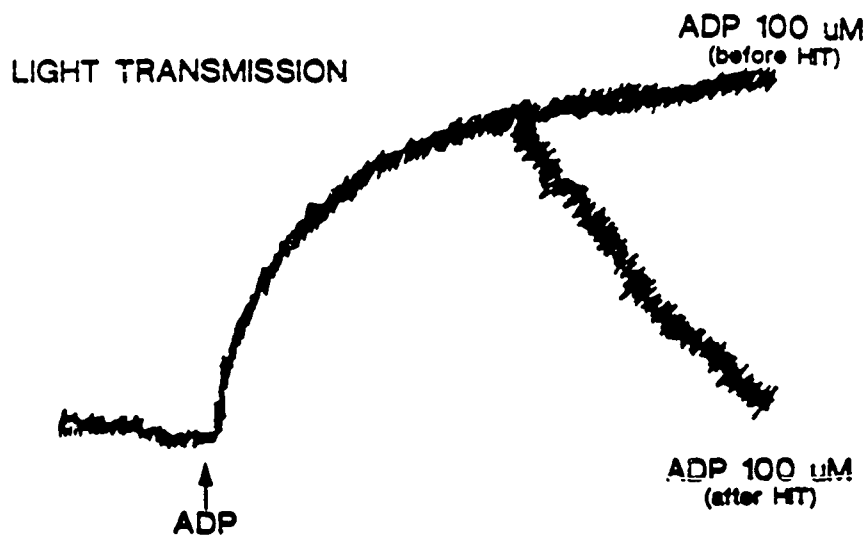


Figure 27.

Platelet diaggregation following HIT administration despite high dose ADP to stimulate aggregation. Platelets behaved normally prior to HIT administration with a dose response relationship between the amount of adenosine diphosphate (ADP) added and the extent of aggregation. However, after HIT, even though the platelets aggregate normally in response to ADP, the platelets disaggregated shortly after ADP stimulated aggregation.

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